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Establishment of the gene transfer system for the primordial cyanobacterium
Gloeobacter violaceus PCC 7421: Alteration of the chlorophyll biosynthetic
pathway by metabolic engineering

Mie Araki

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Abbreviations

ADP	adenosine diphosphate
ALA	5-aminolevulinic acid
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CAO	chlorophyllide <i>a</i> oxygenase
CBB	Coomassie Brilliant Blue
Chl	chlorophyll
Cyt	cytochrome
Fd	ferredoxin
FeS	rieske iron-sulfur cluster
FNR	ferredoxin-NADP ⁺ reductase
HPLC	high performance liquid chromatography
LHC	light-harvesting chlorophyll–protein complex
MES	2-morpholinoethanesulfonic acid, monohydrate
MOPS	3-morpholinopropanesulfonic acid
NADP ⁺	nicotinamide-adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PC	plastocyanin
Pcb	prochlorophyte chlorophyll-binding protein
PCR	polymerase chain reaction
PQ	plastoquinone
PS	photosystem
SDS	sodium dodecyl sulfate

Sm	streptomycin
TES	2-amino-2-(hydroxymethyl)-1,3-propanediol
TRFS	time-resolved fluorescence spectra
Triton X-100	polyoxyethylene p-t-octylphenyl ether

Chapter 1

General introduction

1-1. Significance of photosynthesis research

Light energy is converted to chemical energy by photosynthesis, and the converted chemical energy is used for carbon fixation. Additionally, oxygen is evolved by oxygenic photosynthetic organisms. The fixed carbon dioxide and evolved oxygen support the life of almost all organisms on the earth. Recently, the problems such as global warming caused by rise of carbon dioxide, energy shortage by depletion of a fossil fuel and food shortage are pointed out. These problems should be solved as soon as possible. At the same time, the elucidation of carbon fixation and energy conversion mechanisms is considered to be helpful for both maintenance of global environment and development of the method for acquiring new energy.

It is estimated that oxygen hardly existed in the primitive earth and accumulated by the function of cyanobacteria emerged more than about 2.7 billion years ago. It is suggested that the environment of earth was changed into oxidative from reductive, and the ecosystem was drastically changed by prosperity of cyanobacteria (Summons et al. 1999). Hence, photosynthesis has important roles for the evolution of organisms, maintenance of global environment and various phenomena.

1-2. Classification of photosynthetic organisms

Photosynthetic organisms are classified into prokaryotic organisms and eukaryotic organisms based on presence or absence of nucleus (Fig. 1). Prokaryotic organisms are divided into anoxygenic photosynthetic organisms (photosynthetic bacteria) and oxygenic photosynthetic organisms (cyanobacteria including prochlorophytes) based on the difference in the electron donor used for photosynthesis. Meanwhile, all eukaryotic photosynthetic organisms are oxygenic photosynthesis organisms like cyanobacteria. Eukaryotic photosynthetic organisms are classified into two groups, organisms emerged by primary

endosymbiosis and organisms originated from secondary endosymbiosis. The former was born by the incorporation of an ancestral cyanobacterium into non-photosynthetic eukaryotic organism, and Rhodophyta, Chlorophyta and Glaucocystophyta are considered to be primary endosymbiotic organisms. The incorporated cyanobacterium became chloroplasts during evolution. Secondary endosymbiosis occurred by the incorporation of the eukaryotic photosynthetic organism into non-photosynthetic eukaryotic organism, and Euglenophyta, Chromophyta and Dinophyta are secondary endosymbiotic organisms. Further endosymbiosis may occur for a part of algae.

Table 1 shows the feature of photosynthetic bacteria, cyanobacteria, algae and plants. Oxygenic photosynthetic organisms consist of cyanobacteria and all eukaryotic photosynthetic organisms. Photosynthetic bacteria use hydrogen sulfide, molecular sulfur and organic acids and thiosulfate as electron donor of photosynthetic reaction depending on species. Therefore, photosynthetic bacteria cannot evolve molecular oxygen. In contrast, oxygenic photosynthetic organisms use water molecule as an electron donor of photosynthetic reaction, and oxygen is evolved as the result. Electron transfer system of oxygenic photosynthetic organisms consist of two pigment-protein complexes referred to as photosystem (PS) I and PS II. PS I and PS II are supercomplex. PS I consists of more than ten subunits and PS II consists of more than twenty subunits (Amunts et al. 2007, Umena et al. 2011). The electron transfer system of photosynthetic bacteria consists of only one PS. PSs of green sulfur bacteria and heliobacteria share homology with PS I of oxygenic photosynthetic organisms. Contrary, PSs of green filamentous bacteria and purple bacteria are homologous with PS II of oxygenic photosynthetic organisms (Olson 1981). The photosynthetic bacteria use bacteriochlorophylls as photosynthetic pigments, whereas oxygenic photosynthetic organisms use chlorophyll (Chl) as photosynthetic pigments. Hereafter, I mainly deal with the oxygenic photosynthetic organisms, especially cyanobacteria.

1-3. Photosynthetic electron-transfer reaction of oxygenic photosynthetic organisms

1-3-1. Outline of photosynthetic electron-transfer reaction

Photosynthetic reaction classified into light reaction and dark reaction. The light reaction is the reaction that converts light energy into chemical energy, and the dark reaction is the reaction that produces carbohydrates from carbon dioxide using the chemical energy. In photosynthetic reaction of oxygenic photosynthetic organisms, the photosynthetic apparatus involved in the light reaction exists in the photosynthetic membranes called thylakoid membranes. Figure 2 shows overall electron transfer pathway in cyanobacteria. The constituents of photosynthetic electron transport are PS II, plastoquinone (PQ), cytochrome (Cyt) *b₆f* complex, plastocyanin (PC), PS I, ferredoxin (Fd), Fd-NADP⁺ reductase (FNR) and NADP⁺. The photosynthetic electron transport reaction is started from the charge separation of Chl in PS II reaction center. In the lumenal side of the reaction center, Mn-Ca cluster oxidizes two molecules of water and generates four electrons, four protons and one oxygen molecule. The electrons from water are supplied from the Mn-Ca cluster to the oxidized special Chl *a* pair (P680) of PS II reaction center. From P680, electron is finally transferred to PQ through electron transfer components in PS II. PQ receives two electrons, and the reduced PQ (PQH₂) transfers electrons to Cyt *b₆f*. At this time, H⁺ is transported from stromal side to lumenal side. In Cyt *b₆f*, the electron is transferred to a Rieske iron-sulfur cluster (2Fe-2S), and the electron is further transferred to PC through the reduction of the Cyt *f*. The reduced PC transfers the electron to oxidized special pair (P700) of PS I. After the charge separation of P700 by light energy, the electron is transferred to Fd through electron transfer components. From the reduced Fd, two electrons are transferred to nicotinamide-adenine dinucleotide phosphate (NADP⁺) to yield reduced nicotinamide-adenine dinucleotide phosphate (NADPH), and this reaction is catalyzed by FNR. The series of reactions form H⁺ concentration gradient

across thylakoid membrane. Adenosine triphosphate (ATP) is synthesized from adenosine diphosphate (ADP) by ATP synthetase using the H^+ gradient mediated as a driving force. The NADPH and ATP synthesized by the light reaction are used for carbon dioxide fixation in the dark reaction (Nugent 1996, Ivanov and Khorobrykh 2003).

1-3-2. Features of the photosynthetic system in oxygenic photosynthetic organisms

The protein composition of reaction center of PS I and PS II, primary electron donor, primary electron acceptor, terminal electron acceptor and terminal electron donor are basically common in oxygenic photosynthesis organisms. However, major differences are found in peripheral antenna system. Figure 3 shows the constitution of peripheral antenna systems. Cyanobacteria and Rhodophyta have a supercomplex called phycobilisome that contains phycobilins as antenna pigments (Edwards and Gantt 1971, Koller et al. 1977). In contrast, Chlorophyta and plants have antenna called light harvesting Chl-protein complex (LHC) II inside of membrane (Miller et al. 1976, Brandt et al. 1982, Galetskiy et al. 2008, Damkjaer et al. 2009, Drop et al. 2014). One LHC II binds 12 or 13 Chls (Kühlbrandt et al. 1994). For the structure of thylakoid membranes, grana is found in the thylakoid membranes in plants, however, cyanobacteria do not form the grana. Contrary to the difference in the structure of thylakoid membranes and antenna system, photosynthetic reaction centers are highly conserved from cyanobacteria to higher plants. Therefore, cyanobacteria are suggested to be the model of plants for clarifying the mechanism of photosynthesis.

1-4. Photosynthetic pigments

1-4-1. Three types of photosynthetic pigments

Oxygenic photosynthetic organisms have a photosynthetic pigment system to absorb and transfer excitation energy efficiently to the reaction center of PS. There are three kinds of

photosynthetic pigments, Chls, carotenoids, and phycobilins. Although all oxygenic photosynthetic organisms have Chl and carotenoid, phycobilin exists only in some species such as cyanobacteria, Rhodophyta and Glaucocystophyta.

1-4-2. Chlorophylls

Chl is defined as the metal complex of the cyclic tetrapyrrole coordinated Mg. The molecular species of the chlorophyll which is clear by the present are 50 or more kinds, and especially, 20 or more kinds are functioning as a photosynthetic pigment (Tamiaki et al. 2007). Figure 4 shows the chemical structures of several species of chlorophyll that are suggested to functioning in oxygenic photosynthetic organisms. Chl *a* is first isolated, and this is a neutral complex which consists of the divalent magnesium cation divalent and divalent anion of the plane type ligand of four seats. Chl *a*, Chl *b*, Chl *c*, Chl *d*, Chl *f*, [8-vinyl] Chl *a* and [8- vinyl] Chl *b* are suggested to be functioning in natural oxygenic photosynthetic organisms, and they bind to apoprotein and have the function for the light harvesting. Additionally, Chl *a*, Chl *d* and [8- vinyl] Chl *a* are also bearing the function of the charge separation in the reaction center of a photochemistry system besides the light harvesting (Hu et al. 1998, Chisholm et al. 1992, Govindjee and Rabinowitch 1960).

1-4-3. Carotenoids

Carotenoids exist not only in photosynthetic organisms but also in many other organisms, and over 750 carotenoids are distributed in nature. Carotenoid is a compound which consists of isoprene units. The carotenoid of C₄₀ and the derivative are distributed widely. Although the carotenoids associated with photosynthesis are about 150 carotenoids including intermediate products, they are widely distributed depending on organism species (Britton et al. 2004). Carotenoids function in the light harvesting and photoprotection in photosynthesis

(Johnson and Schroeder 1996, Frank and Cogdell 1996) and are combined with apoprotein together with Chls to form the pigment-protein complex.

1-4-4. Phycobilins

Phycobilins consist of an open chain of four pyrrole rings (tetrapyrrole). Phycobilins exist only in Cyanobacteria, Rhodophyta and Glaucocystophyta. The water-soluble phycobilin protein is formed by covalent binding of phycobilin to the cystein residue of apoprotein. Furthermore, it is also known that phycobilin will function as a chromophore of the photoreceptors like phytochrome (Fig. 3). Phycobilin protein forms supercomplex, phycobilisome, which absorbs and supply light energy mainly to the PS II reaction center of cyanobacteria (MacColl 1998).

1-5. A major issue in the photosynthesis research

Although the evolutionary relationship among photosynthetic organisms is not clear, it is suggested that the photosynthetic organism was evolved as summarized in Fig. 5. First, photosynthetic bacteria were born. Some photosynthetic bacteria start to use water as an electron donor, and oxygen evolution type photosynthetic organism (cyanobacteria) emerged. Next, ancient cyanobacterium was incorporated into a eukaryotic organism (primary endosymbiosis) (Tomitani et al. 1999), and the cyanobacteria in the eukaryotic organism became a chloroplast. Then, Rhodophyta, Glaucocystophyta and Chlorophyta were branched. Furthermore, the primary symbionts were incorporated into heterotrophs, and the secondary symbionts were born (secondary endosymbiosis) (Rumpho et al. 2011). Euglenophyta, Chromophyta and Dinophyta belong to the secondary symbionts.

It is suggested that cyanobacteria are the first oxygenic photosynthetic organisms as shown in Fig. 5 and are the ancestors of the chloroplast in the eukaryotic photosynthetic

organisms. Therefore, cyanobacteria are used for research as a model for analyzing the evolution of photosynthetic organisms and mechanism of photosynthesis. The most often used organism is *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*). The complete genome of *Synechocystis* was first sequenced among cyanobacteria (Kaneko et al. 1996). *Synechocystis* are possible to grow not only under the photoautotrophic conditions but also under the heterotrophic conditions using glucose by photoactivation (Joset et al. 1988). Additionally, *Synechocystis* can be manipulated using homologous recombination (Vermaas 1996). Hence, molecular genetic analysis of *Synechocystis* proceeded. Almost all the oxygenic photosynthetic organisms including *Synechocystis* form thylakoid membranes that contain photosynthetic electron transport system. In the oxygenic photosynthetic organisms, most of respiratory system exists in cell membrane, and the little of them is colocalized with photosynthetic apparatus in the thylakoid membranes. Assuming the oxygenic photosynthetic organism was evolved from anoxygenic photosynthetic organisms (Fig. 5), the early oxygenic photosynthetic organism was estimated to lack thylakoid membrane, and photosynthetic electron transport system and respiratory system existed on the same membrane. Therefore, in order to understand the mechanism of the photosynthetic system in the early oxygenic photosynthetic organism and the meaning of thylakoid membrane formation, it is necessary to analyze organism that lacks thylakoid membrane.

Almost all cyanobacteria and Rhodophyta have Chl *a*, carotenoids and phycobilins as photosynthetic pigments, while some cyanobacterial species including Prochlorophytes that are seemed to be losing phycobilisome also contain Chl *b* or Chl *d* or [8- vinyl] Chls. In contrast, Chlorophyta and plants have Chl *b* in addition to Chl *a* without phycobilins. Regarding the difference in the pigment composition among oxygenic photosynthetic organisms, Tomitani et al. (1999) proposed that the common ancestor of cyanobacteria and chloroplasts contained both Chl *b* and phycobilins in addition to Chl *a*. This hypothesis

suggested that Chl *b* has been lost in the lineage of Rhodophyta and Glaucocystophyta, and phycobilins have been lost in the lineage of Chlorophyta and Prochlorophytes during the course of evolution (Tomitani et al. 1999). However, the photosynthetic organisms similar to the common ancestor hypothesized by Tomitani et al. (1999) have not been found in nature.

1-6. Characteristics and research problem of *Gloeobacter violaceus* PCC 7421

A unicellular cyanobacterium, *Gloeobacter violaceus* PCC 7421 (hereafter referred to as *G. violaceus*) was first isolated from the surface of limestone rock in Switzerland in 1974 (Rippka et al. 1974). Molecular phylogenetic analyses indicate that *G. violaceus* is an early-branching cyanobacterium within cyanobacterial clade (Nelissen et al. 1995). *G. violaceus* is the only known oxygenic photosynthetic organism that lacks the thylakoid membranes. Therefore, the places of both the photosynthetic electron transport system and the respiratory system, which are mostly divided in other cyanobacteria and eukaryotic photosynthetic organisms, exist on the cell membrane in *G. violaceus* (Rippka et al. 1974). This means that periplasmic side instead of lumenal side is the site of the oxygen evolution in *G. violaceus* (Rippka et al. 1974). The doubling time of *G. violaceus* was approximately 73 h in liquid culture, and this is later compared with *Synechocystis*, a model organism of cyanobacteria. In addition, the suitable light intensity for culturing *G. violaceus* is less than 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ that is much lower than that for *Synechocystis* (Rippka et al. 1974). The complete genome of *G. violaceus* was sequenced in 2003 (Nakamura et al. 2003). Generally, the subunits of PSs are mostly conserved from cyanobacteria to higher plants. However, *G. violaceus* lacks several genes related to photosynthesis. For example, *psaI*, *psaJ* and *psaK* for PS I, *psbY*, *psbZ* and *psb27* for PS II were not found (Nakamura et al. 2003). In our laboratory, the following features were demonstrated by using *G. violaceus* wild type; a novel subunit named PsaZ was found in PS I instead of PsaI, PsaJ and PsaK (Inoue et al. 2004), the

phycobilisomes form a unique structure with novel linker proteins (Koyama et al. 2006), the bonding pattern of extrinsic proteins (PsbO, PsbU, PsbV) with CP47 and CP43 of PS II core complex differed from that of other cyanobacteria (Koyama et al. 2008). Recently, Rexroth et al. (2011) indicated that photosynthetic region and other region are separated in cell membrane of *G. violaceus*. Contrary to the increase of biochemical characterization of *G. violaceus* with interesting features, the lack of molecular genetic analysis prevented us from further understanding of *G. violaceus*.

1-7. The aim of this study

There is long history in the research on the molecular mechanism of photosynthetic reaction. To clarify the mechanism, the physiological, biochemical, biophysical and molecular genetic methods are used. Especially, molecular genetic method is a powerful tool and essential for elucidating the function of each gene. Therefore, the aim of this study is to establish the transformation system for *G. violaceus*.

G. violaceus is estimated to be an early-branching cyanobacterium within the cyanobacterial clade by the molecular phylogenetic analysis (Nelissen et al. 1995), and lacks thylakoid membrane (Rippka et al. 1974). Therefore, I estimated that *G. violaceus* is expected to be a model organism of ancestral cyanobacterium whose PSs and respiratory chain might be localized in cell membrane because of the lack of thylakoid membranes. The analyses of oxygen evolution system and the interaction of the photosynthetic electron transport system and the respiratory system in *G. violaceus* will reveal the unique features. Comparing them with those of other cyanobacteria, basic principles of photosynthetic system and the evolution of photosynthesis from the early oxygenic photosynthetic organism are expected to be partly solved. To date, spectrophotometric and biochemical analyses using wild type *G. violaceus* have been reported. However, the molecular genetic analysis (gene targeting, foreign gene

expression and comprehensive gene disruption) is needed for further analysis of *G. violaceus*. There is only one report on the development of the transformation system for *G. violaceus* (Guo and Xu 2004), however, no further study using the system has been reported.

In this study, I developed the transformation system for *G. violaceus* (Chapter 2), and altered the Chl biosynthetic pathway by metabolic engineering using the developed system (Chapter 3). This study consists of two experimental parts (Chapters 2 and 3) and one summary and perspective part (Chapter 4).

Chapter 2

Establishment of the reporter system for *Gloeobacter violaceus*

PCC 7421

Summary

Gloeobacter violaceus PCC 7421 is considered, by molecular phylogenetic analyses, to be an early branching cyanobacterium within the cyanobacterial clade. *G. violaceus* is the only known oxygenic photosynthetic organism that lacks thylakoid membranes. There is only one report on the development of a transformation system for *G. violaceus* [H. Guo, X. Xu, Prog. Nat. Sci. 14 (2004) 31–35] and further studies using the system have not been reported. In this chapter, I succeeded in introducing an expression vector (pKUT1121) derived from a broad-host-range plasmid, RSF1010, into *G. violaceus* by conjugation. The frequency of transformation of my system is significantly higher than that described in the previous report. In addition, luciferase heterologously expressed in *G. violaceus* functioned as a reporter. The established system will promote the molecular genetic studies on *G. violaceus*.

2-1. Introduction

Cyanobacteria are considered to be the first oxygenic photosynthetic organisms that emerged about 2.7 billion years ago. Most of the genes that are responsible for photosynthesis are widely conserved from cyanobacteria to eukaryotic photosynthetic organisms, this conservation is a convincing evidence of the endosymbiotic acquirement of eukaryotic chloroplast from a cyanobacterium. This high conservation has prevented us from understanding of the evolution of photosynthetic mechanisms from the primordial one. Therefore, cyanobacteria diverged from early stage of the cyanobacterial evolution may be helpful in studying the evolution of photosynthetic mechanisms, because such cyanobacteria are expected to retain a part of primordial properties that had been lost during the evolution of other major cyanobacteria. However, “primordial cyanobacteria” that retain a part of primordial properties rarely exist nowadays. *Gloeobacter violaceus* PCC 7421 is a unicellular cyanobacterium, and is considered to be an early-branching cyanobacterium within the

cyanobacterial clade, by the molecular phylogenetic analyses (Nelissen et al. 1995, Swingley et al. 2008, Falcón et al. 2010, Gupta and Mathews 2010). Almost all oxygenic photosynthetic organisms form the internal membranes called thylakoid membranes, which are the site for the light reaction of photosynthesis. *G. violaceus* is the only known oxygenic photosynthetic organism that lacks the thylakoid membranes (Rippka et al 1974). This unique property has been found only in this organism. Accordingly, both the photosystems and the respiratory chain in *G. violaceus* are localized at the cell membrane. This indicates that photosynthetic activity per cell in *G. violaceus* is much lower than those in other cyanobacteria and eukaryotic photosynthetic organisms. For these unique characteristics, the complete genome of *G. violaceus* was sequenced in 2003 (Nakamura et al. 2003). The genome sequence revealed that a part of the genes that are responsible for photosynthesis was not found in *G. violaceus*, whereas those genes are highly conserved among other oxygenic photosynthetic organisms (Nakamura et al. 2003). Therefore, in recent years, protein complexes that are responsible for photosynthesis (e.g. photosystem I and phycobilisome) in *G. violaceus* were biochemically analyzed based on the genome information (Inoue et al. 2004, Koyama et al. 2006, Sicora et al. 2008, Dreher et. at. 2010, Mendoza-Hernández et al. 2010). These recent results partly solved unique features previously reported (Guglielmi et al. 1981, Koenig and Schmidt 1995, Mangels et al. 2002). Recently, it was reported that both the photosynthetic and respiratory complexes were concentrated at the respective domains, which may have specialized functions, in the cell membrane of *G. violaceus* (Rexroth et al. 2011). Moreover, the comparison of state transitions between *G. violaceus* and *Synechocystis* showed the commonalities and differences (Bernát et al. 2012). *G. violaceus* exhibited state transitions and non-photochemical fluorescence quenching like *Synechocystis* (Bernát et al. 2012). In *G. violaceus*, the structure of phycobilisome was quite different from other cyanobacterial phycobilisomes (Koenig and Schmidt 1995). Nevertheless, orange carotenoid

protein that binds to phycobilisome was also correlated with blue-light-induced heat dissipation in *G. violaceus*, like *Synechocystis* (Bernát et al. 2012). These results suggest that *G. violaceus* is an ideal organism for investigating the evolution of photosynthetic system by comparison of other cyanobacteria. Molecular genetics, such as the production and analysis of mutants, is a preferable method to analyze the function of individual genes in *G. violaceus*. Unfortunately, molecular genetic analysis cannot be applied to *G. violaceus* because of the lack of a highly-reproducible transformation system for this organism. Only one report on the development of a transformation system for *G. violaceus* has been published to date (Guo and Xu 2004). However, there is no subsequent paper that describes the functional expression of the foreign genes in *G. violaceus* using the system.

As techniques used for transformation of cyanobacteria, there are natural transformation, electroporation and conjugation. Figure 6 shows schematic of each technique. The natural transformation is a method that DNA is introduced into a cell, when DNA such as plasmid and cyanobacteria are mixed (Fig. 6A). The successful examples have been reported *Synechocystis* (Yoshihara et al. 2001), *Thermosynechococcus elongatus* BP-1 (Onai et al. 2004), *Synechococcus elongatus* PCC 7942 (Golden et al. 1984) and *Synechococcus* sp. PCC 7002 (Frigaard et al. 2004). However, the species which can be transformed by natural transformation is restricted. Electroporation is the method that subjecting the cells to high field strength electrical pulses causes reversible transient pores in the cell membrane through which DNA can enter (Fig. 6B). Electroporation is a method widely used for transformation. It has succeeded in *Synechocystis* sp. (Zang et al. 2007), *T. elongatus* BP-1 (Katoh et al. 2001) and other unicellular cyanobacteria, and *Anabaena* sp. (Thiel et al. 1989) and other filamentous cyanobacteria. Transformation by the electroporation requires dedicated system. Conjugation is the transfer of plasmid from *E. coli* to cyanobacterium by cell-to-cell contact (Fig. 6C). Conjugation is used for *Synechocystis* sp. (Mermet-Bouvier et al. 1994),

Synechococcus sp. (Tsinoremas et al. 1994), *T. elongatus* BP-1 (Mühlenhoff and Chauvat 1996), *Acaryochloris marina* MBIC 11017 (Tsuchiya et al. 2012B), *Anabaena* sp. (Wolk et al. 1984), *Nostoc* PCC 6310 (Flores and Wolk 1985) and other cyanobacteria. Transformation by the conjugation requires the plasmid which has a gene cluster required for conjugal transfer and selection of a vector with origin of conjugal transfer. However, transformation system of many organisms is not developed because of the barrier by the restriction system and difficulty of cultivation. In cyanobacteria, molecular genetics analysis using the transformation system of Fig. 7 has been conducted using the above three transformation technique. Figure 7A is the transformation system for expression of target gene by transgenic. Figure 7B is the gene targeting system for change or destruction of a target gene (reverse genetics). Figure 7C is a system which disrupts a gene comprehensively and selects a transformant from a phenotype, and specifies the causative gene (forward genetics). Therefore, it is necessary to develop these three experimental systems for *G. violaceus*. In this study, I decided to develop the transformation system corresponding to Fig. 7A. First, I re-examined the transformation system reported previously, and developed a highly reproducible transformation system for *G. violaceus*. I succeeded in introducing an expression vector derived from a broad-host-range plasmid into *G. violaceus* by conjugal gene transfer. Using this system, I introduced a luciferase gene into *G. violaceus*, and the resultant transformant exhibited significant luciferase activity.

2-2. Materials and Methods

2-2-1. Culture of *G. violaceus*

G. violaceus was grown photoautotrophically in BG11 medium (Allen 1968) under the continuous white light ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 25°C, and air was supplied via an air filter (Millex-FG, Millipore, Massachusetts, USA). For transformants, $10 \mu\text{g ml}^{-1}$

streptomycin (Sm) was added to the medium. BG11 agar medium containing 1 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TES)-NaOH (pH 8.2) and 5 $\mu\text{g ml}^{-1}$ Sm was used for solid culture.

2-2-2. Construction of plasmids and transformation of *G. violaceus*

I used a plasmid vector pKUT1121 (Fig. 8) (Tsuchiya et al. 2012B), which was constructed from a broad-host-range plasmid RSF1010 (Scholz et. al. 1989), to establish a transformation system for *G. violaceus*. The coding region of firefly luciferase gene (*luc*) was amplified by polymerase chain reaction (PCR) using pGL3-Basic vector (Promega, Wisconsin, USA) as a template. The PCR product containing *luc* gene with additional restriction sites for *NdeI* and *XhoI* at the 5'- and 3'- ends, respectively, was amplified using the Table 2 lucFw and lucRv primers. After the PCR product was subcloned into pZErO-2 (Invitrogen, California, USA), the sequence of cloned *luc* gene was confirmed by sequencing. The *luc* gene was excised from the plasmid by *NdeI* and *XhoI* treatment, and subcloned into pKUT1121 to yield pKUT1121-*luc* (Fig. 9). Transformation was performed by diparental mating basically according to the method of Elhai and Wolk (Fig. 6C) (Elhai and Wolk 1988). First, a conjugative helper plasmid, pRK2013 (Figurski and Helinski 1979), was introduced into *Escherichia coli* XL1-Blue MRF' (Agilent Technologies, California, USA). Subsequently, the expression vector (pKUT1121 or pKUT1121-*luc*) was introduced into XL1-Blue MRF' (pRK2013). Equal amounts of resultant transformant cells and *G. violaceus* cells were mixed, and then aliquots of the mixture were spotted onto nitrocellulose membrane on a BG11 agar medium. Following a 48 h incubation under the light of 5 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, the membrane was transferred onto BG11 agar medium containing 5 $\mu\text{g ml}^{-1}$ Sm. Sm-resistant colonies appeared after several months, and each colony was finally cultured in BG11 liquid medium containing 10 $\mu\text{g ml}^{-1}$ Sm. Total DNA was prepared from *G. violaceus* cells using

hexadecyl-trimethyl-ammonium bromide (Wilson 1997). The presence of marker gene in the total DNA was checked by PCR.

2-2-3. Polymerase chain reaction

I confirmed successful introduction of the plasmid into *G. violaceus* by PCR. The *aadA* gene was amplified using the Table 2 primer SmFw and SmRv, the *luc* gene was amplified using the Table 2 primer lucFw and lucRv. The enzyme of PCR used *Ex Taq* Hot Start Version (Takara, Siga, Japan). The equipment for a reaction used thermal cycler (Biorad, California, U.S.A), and reaction conditions were shown in Table 3.

2-2-4. Preparation of total protein from G. violaceus

Total protein of *G. violaceus* cells was prepared by the following procedure. *G. violaceus* was resuspended with a buffer (20 mM 2-morpholinoethanesulfonic acid, monohydrate (MES)–NaOH (pH 6.5), 1 mM MgCl₂, 0.5 mM CaCl₂, 1 mM NaCl, 0.6 M betaine). The suspended cells were disrupted by repeated agitation with glass beads (ϕ = 0.1 mm) at 4°C. After the debris was removed by centrifugation (2000 × g, 5 min, 4°C), polyoxyethylene p-t-octylphenyl ether (Triton X-100) was added to the supernatant at the final concentration of 1% to solubilize the membrane. The prepared total protein was quantitated by bicinchoninic acid assay (Smith et al. 1985), and the standard solution used bovine serum albumin.

2-2-5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (Laemmli 1970) using 12% (w/v) of polyacrylamide gel. Total proteins (10 µg) were loaded on each lane of a gel, and the gel was stained with Coomassie Brilliant

Blue (CBB) after electrophoresis. For Western blotting, separated proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, New Jersey, USA). Western blotting and chemiluminescence detection were performed according to the manufacturer's instructions. Anti-luciferase antibody (Luciferase (251-550), Santa Cruz Biotechnology, California, USA) was used as a primary antibody. After the treatment with secondary antibody (Anti-Rabbit IgG, Jackson Immuno Research Europe, Suffolk, UK), luciferase was detected by chemiluminescence (ECL Plus Western Blotting Detection System, GE Healthcare) with a luminescent image analyzer (LAS-3000 UV mini, Fujifilm, Tokyo Japan).

2-2-6. Reporter assay using firefly luciferase

The concentration of *G. violaceus* cells was adjusted to 1.0×10^7 cells ml⁻¹ with BG11 medium. After the cells were adapted to darkness for 5 min, background luminescence was measured with a luminometer (GloMaxTM 20/20n Luminometer, Promega). Then, luciferin (Beetle Luciferin, Promega) was added to the cells at the final concentration of 100 µM, and the luminescence derived from luciferase reaction was measured.

2-3. Results

2-3-1. Antibiotic susceptibility of G. violaceus

First, I tried to culture *G. violaceus* at 28°C under the light of 15 µmol photons m⁻² s⁻¹ according to Guo and Xu (Guo and Xu 2004), however, cells were not able to survive (Fig. 10A). In contrast, cells were able to grown by laboratory condition (25°C under the light of 5-10 µmol photons m⁻² s⁻¹) (Fig. 10B). Therefore, I applied our routine culture conditions to further study. I examined the antibiotic susceptibility of *G. violaceus* for the use of antibiotic resistance genes as marker genes of transformant. I tested gentamicin, hygromycin,

spectinomycin and zeocin in addition to antibiotics used in Guo and Xu (Guo and Xu 2004). Table 4 summarizes the result of antibiotic susceptibility test of wild type *G. violaceus*. Three antibiotics showed same susceptibility as described in Guo and Xu (Guo and Xu 2004), however, the others showed different susceptibility (for details, see Section 2-4). Three of nine antibiotics, erythromycin, Sm and spectinomycin exhibited antibiotic activity against *G. violaceus* within the range of 1-50 $\mu\text{g ml}^{-1}$. For these three antibiotics, I also checked the antibiotic activity against *G. violaceus* on the agar medium. *G. violaceus* cells adjusted to the concentration of 1.0×10^3 to 1.0×10^9 cells ml^{-1} were spotted onto nitrocellulose membrane on BG11 agar medium including each antibiotic (Table 5). As a result, Sm was the most effective for killing cells at lower concentration (5 $\mu\text{g ml}^{-1}$). Therefore, I chose Sm resistance gene (*aadA*) as a marker gene for the screening of transformant.

2-3-2. Development of the transformation system for *G. violaceus*

Because *G. violaceus* was sensitive to Sm (Table 5), I tried to introduce a broad-host-range plasmid derived expression vector, pKUT1121 (Tsuchiya et al. 2012B) that possesses Sm resistance gene cassette, by conjugal gene transfer. After the treatment of exconjugants with Sm, Sm resistant colonies appeared (Fig. 11A). In contrast, no colony was formed in the spot of negative control (Fig. 11B). The frequency of transformation of *G. violaceus* was approximately 1.2×10^{-4} per recipient cell for pKUT1121. Total DNA prepared from the Sm resistant strain and wild type were used as template of PCR (Fig. 12) to confirm successful introduction of the plasmid. As a marker gene, *aadA* was amplified by PCR (Fig. 12A), and a PCR product that exhibited the similar migration to that of positive control (Fig. 12B, lane 2) was observed in the Sm resistant strain (Fig. 12B, lane 3) by electrophoresis. In contrast, no amplification was found in wild type (Fig. 12B, lane 1). Furthermore, I transformed two strains of *E. coli* (XL1-Blue MRF' and DH5 α) with the total

DNA. For each *E. coli* strain, a lot of Sm resistant colonies appeared after the transformation with the total DNA prepared from Sm resistance strain. On the contrary, no colony formed after the transformation with the total DNA from wild type. Then, plasmids prepared from the *E. coli* transformants were digested with restriction enzymes. In agarose gel electrophoresis (Fig. 13), the restriction patterns of the prepared plasmids (Fig. 13, lanes 4-9) were both introduced DH5 α (Fig. 13B) and XL1-Blue MRF' (Fig. 13C) identical to that of the original pKUT1121 (Fig. 13A). These results demonstrated that the Sm resistant strain harbors pKUT1121 as a plasmid. Therefore, I concluded that the transformation system for *G. violaceus* was established. I named the *G. violaceus* transformant pKUT1121 strain, and used this strain as a control for further experiments.

2-3-3. Introduction of a *luc* gene into *G. violaceus* as a reporter gene

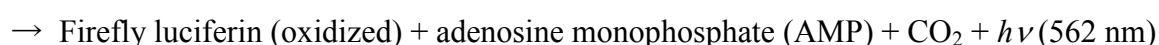
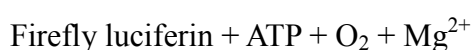
Because I succeeded in developing the transformation system for *G. violaceus* by conjugation, I evaluated the use of a *luc* gene as a reporter gene in *G. violaceus* cells. The *luc* gene was subcloned into pKUT1121, and the resultant vector (pKUT1121-*luc*, Fig. 9) was introduced into *G. violaceus*. By conjugation, Sm resistant colonies were obtained at the frequency of transformation of approximately 3.6×10^{-5} per recipient cell. Total DNA was prepared from this Sm resistant strain (pKUT1121-*luc* strain), and used as a template of PCR. Two marker genes, *aadA* and *luc* were successfully amplified (Fig. 14A) by PCR at total DNA of pKUT1121-*luc* strain (Figs. 14B and 14C; lane 4) as same as original plasmid (Figs. 14B and 14C; lane 3), whereas *luc* gene was not amplified from the total DNA of both wild type and pKUT1121 strain (Figs. 14B and 14C; lanes 1 and 2). Transformation of *E. coli* with the total DNA prepared from pKUT1121-*luc* strain confirmed that pKUT1121-*luc* (Fig. 15, lanes 3 and 4) was maintained as a plasmid in pKUT1121-*luc* strain (Fig. 15, lanes 1 and 2).

2-3-4. Detection of luciferase in the pKUT1121-luc strain by Western blotting

I examined whether the *luc* gene was functionally expressed in the pKUT1121-*luc* strain, by Western blotting and luciferase assay. Total proteins prepared from cells were analyzed by SDS-PAGE and following Western blotting. Although no specific band was found among wild type, pKUT1121 strain and pKUT1121-*luc* strain by CBB staining of the gel (Fig. 16A), luciferase was immunochemically detected only in the pKUT1121-*luc* strain (Fig. 16B).

2-3-5. Activity of luciferase expressed in the pKUT1121-luc strain

For wild type and transformants, I measured luciferase activity *in vivo* (Table 6). The following chemiluminescence reaction is catalyzed by firefly luciferase;



The background luminescence of each sample was measured after the dark adaptation of the cells, and the values were quite low in all samples. After the addition of luciferin to the cells, approximately 1000 times higher luminescence than background was observed in pKUT1121-*luc* strain, whereas virtually no difference was found in wild type and pKUT1121 strain.

2-4. Discussion

Development of the transformation system for *G. violaceus* was already reported by Guo and Xu (Guo and Xu 2004). They demonstrated the introduction of a RSF1010 derived plasmid into *G. violaceus* by conjugation. However, I could not reproduce even their culture condition (28–30°C, 10–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Because *G. violaceus* was reported to be a cyanobacterium that can grow only under the dim light (Rippka et al. 1974), I presumed that *G. violaceus* used by Guo and Xu (Guo and Xu 2004) was adapted to slightly stronger light

condition during long cultivation. Since the higher-light adapted *G. violaceus* was not obtained in my experiment, I examined proper conditions suitable for the transformation of *G. violaceus* using my routine culture. Sensitivity of *G. violaceus* to erythromycin and Sm (Table 4) was similar to that described in Guo and Xu (Guo and Xu 2004). However, sensitivity to ampicillin and chloramphenicol was different from the former result, which demonstrated that ampicillin was effective at the concentration of $15 \mu\text{g ml}^{-1}$ and chloramphenicol was ineffective up to $100 \mu\text{g ml}^{-1}$ (Guo and Xu 2004). I newly evaluated the four antibiotics (gentamicin, hygromycin, spectinomycin and zeocin) for *G. violaceus*. Among them, only spectinomycin was effective. I used expression vectors derived from a broad-host-range plasmid RSF1010, because *G. violaceus* have no endogenous plasmid (Nakamura et al. 2003). Guo and Xu (Guo and Xu 2004) reported that no transformant was obtained by the transformation of *E. coli* DH5 α with DNA prepared from plasmid introduced *G. violaceus* whereas transformants appeared with high efficiency in the case of a methylation-restriction mutant, DH10B as a host strain. They concluded that the difference in the transformation efficiency reflect the presence of DNA methylation in *G. violaceus*, which caused the restriction by *E. coli*. On the other hand, my results using DH5 α and a methylation-restriction mutant, XL1-Blue MRF' demonstrated that there is no difference in the transformation efficiency of two strains. Guo and Xu (Guo and Xu 2004) used a vector, pKT210 (Bagdasarian et al. 1981), which is different from I used vector, pKUT1121 that was recently constructed (Tsuchiya et al. 2012B). Both vectors are derived from a broad-host-range plasmid RSF1010, however, pKUT1121 is 3.3 kb smaller than pKT210. Therefore, the transformation efficiency of pKUT1121 might be higher than that of pKT210 for *E. coli*. However, I assumed that the difference is not related to DNA methylation in *G. violaceus* because DH10B was transformed with high efficiency using pKT210 (Guo and Xu 2004). Hence, this inconsistency also indicated that the properties of *G. violaceus* reported in 2004

were quite different from our laboratory strain. In the transformation of bacteria, restriction-modification system in the cell affects the frequency of transformation. In the case of *Anabaena* sp. PCC 7120, type II DNA restriction-modification system is major barrier against transformation (Elhai and Wolk 1988). This problem was overcome by the coexpression of DNA methylases that were associated with type II restriction enzymes in the *E. coli* (Elhai and Wolk 1988). Guo and Xu (Guo and Xu 2004) reported that the frequency of transformation of *G. violaceus* raised from 4.63×10^{-6} to 1.67×10^{-5} by the coexpression of three DNA methylases that were effective for the transformation of *Anabaena* (Elhai et al. 1997). In my result, the frequency of transformation of pKUT1121 strain was 1.2×10^{-4} without coexpression of *Anabaena* DNA methylases. The frequency of transformation of my system is significantly higher than that described in the previous report. Moreover, no genes for type II restriction-modification system were found in the genome of *G. violaceus* (Nakamura et al. 2003). These results indicate that the barrier of the restriction system for transformation is not high in *G. violaceus* unlike *Anabaena*. The luciferase assay revealed that there is no significant background activity in *G. violaceus* (Table 6). Therefore, the *luc* gene can be available for promoter assay using *G. violaceus* as a host. Although I could not succeed in accumulating luciferase at a high level (Fig. 16), this low-level expression will be enough for the metabolic engineering of *G. violaceus*. The genes for enzymes that are responsible for chlorophyll, carotenoid and lipid biosynthesis are candidates for alteration, because those molecules affect photosynthetic activity. In the present study, I established the highly-reproducible transformation system on *G. violaceus*, and demonstrated the introduction and functional expression of the *luc* gene in *G. violaceus*. Using my system, other molecular genetic techniques such as transposon tagging and gene targeting will be developed in the future, and the analyses of *G. violaceus* will progress by the novel techniques.

Chapter 3

**Artificially acquired chlorophyll *b* is highly acceptable to
Gloeobacter violaceus PCC 7421**

Summary

Unicellular cyanobacterium *Gloeobacter violaceus* is an only known oxygenic photosynthetic organism that lacks thylakoid membrane. Molecular phylogenetic analyses indicate that *G. violaceus* is an early-branching cyanobacterium within cyanobacterial clade. Therefore, the photosynthetic system of *G. violaceus* is considered to be partly similar to that of the ancestral cyanobacteria that would lack thylakoid membrane. *G. violaceus* possesses chlorophyll (Chl) *a* as the only chlorophyll species like most cyanobacteria. It was proposed that the ancestral oxygenic photosynthetic organism had not only Chl *a* and phycobilins but also Chl *b*. However, no organism which contains both Chl *a* and Chl *b* and lacks thylakoid membrane has been found in nature. Therefore, I introduced the chlorophyllide *a* oxygenase gene responsible for Chl *b* biosynthesis into *G. violaceus*. In the resultant transformant, Chl *b* accumulated at approximately 11% of total Chl independent of growth phase. Photosystem I complexes isolated from the transformant contained Chl *b* at 9.9% of total Chl. The presence of Chl *b* in the photosystem I complexes did not inhibit trimer formation. Furthermore, time-resolved fluorescence spectrum demonstrated that Chl *b* transferred energy to Chl *a* in the photosystem I complexes and did not disturb the energy transfer among the Chl *a* molecules. These results show that *G. violaceus* is tolerant to artificially produced Chl *b* and suggest the flexibility of photosystem for Chl composition in the ancestral oxygenic photosynthetic organism.

3-1. Introduction

Light energy is converted into chemical energy by photosynthesis. In the photosynthetic organisms, photochemical reaction occurs by the pigment-protein complex called photosystem (PS). Subunit constitution of photosystems are highly conserved among oxygenic photosynthetic organisms, and almost all species possess chlorophyll (Chl) *a* as the

only Chl species for photochemical reaction. In contrast, Chl species functions in the light-harvesting antenna proteins are diversified; Chl *b*, Chl *c* and Chl *d* are also used as antenna pigments in addition to Chl *a*, depending on organism species. Regarding the light-harvesting antenna proteins, most cyanobacteria, red algae and glaucophytes utilize phycobilisome that contains phycobilins instead of Chl as antenna pigments (Fig. 3). Since the ancestral cyanobacterium is considered to be the origin of chloroplast, studies on the variety and distribution of antenna pigments among cyanobacteria provide meaningful information on the evolution of antenna systems. Among cyanobacteria, there are some exceptional species. Prochlorophytes biosynthesize Chl *b* (or [8-vinyl]-Chl *b*) in addition to Chl *a* (or [8-vinyl]-Chl *a*) (Chisholm et al. 1992, Bullerjahn and Post 1993), and *Acaryochloris* spp. contain Chl *d* as a major Chl (Miyashita et al. 2003). These cyanobacteria use antenna pigment-protein complex, Pcb (prochlorophyte chlorophyll-binding protein), which is homologous to CP43, a major core antenna subunit of photosystem II (van der Staay et al. 1998, Chen et al. 2002, Murray et al. 2006). Phycobiliproteins of these cyanobacteria do not form complete structure of phycobilisome, and the parts of phycobilisome seem to act as antenna. However, Pcb is not phylogenetically related to light-harvesting chlorophyll-protein complexes from Chlorophyta and land plants that contain both Chl *a* and Chl *b*. Considering the distribution of Chl *b* among oxygenic photosynthetic organisms, acquisition of Chl *b* biosynthesis during the evolution of cyanobacteria is one of the attractive issues.

Chl is biosynthesized from glutamic acid through 5-aminolevulinic acid (ALA) (Fig. 17). porphyrinogen is synthesized from condensation of two molecules of ALAs. Four molecules of porphyrinogens are bond, and uroporphyrinogen III is compounded through two steps of reactions. Protoporphyrin IX is synthesized from uroporphyrinogen III through three steps of reactions, such as a decarboxylation of side chain of acetate side and a decarboxylation of the side chain of propionic acid side of A and B ring. The chelate of Mg into protoporphyrin IX is

catalyzed by Mg-chelatase, and Mg-protoporphyrin IX synthesized. Chlorophyllide *a* is synthesized through four steps of reactions from Mg-Protoporphyrin IX. Chl *a* is synthesized from Chlorophyllide *a* (Masuda 2008, Tanaka and Tanaka 2011).

Chl *b* is synthesized from chlorophyllide *a* through chlorophyllide *b*. Chlorophyllide *a* oxygenase (CAO) catalyzes the conversion from chlorophyllide *a* to chlorophyllide *b*. *CAO* gene was first identified from *Chlamydomonas reinhardtii* (Tanaka et al. 1998) and successively cloned from two prochlorophytes and major groups of chlorophytes (Tomitani et al. 1999). The deduced amino acid sequences of coding regions of *CAO* genes showed the existence of the highly conserved region among them. From the molecular phylogenetic analysis of CAOs, Tomitani et al. (Tomitani et al. 1999) proposed that the common ancestor of cyanobacteria and chloroplasts contained both Chl *b* and phycobilins. This hypothesis suggested that Chl *b* has been lost in the lineage of Rhodophyta and Glaucocystophyta, and phycobilins have been lost in the Chlorophyta after the primary endosymbiosis (Tomitani et al. 1999). In the previous research, Chl *b* was artificially produced in a cyanobacterium that could not synthesize Chl *b*. *Arabidopsis CAO* cDNA was introduced into the genome of *Synechocystis*, and the resultant transformant accumulated Chl *b* (Satoh et al. 2001). For the transformant, energy transfer from Chl *b* to Chl *a* was indicated by the spectroscopic measurements of cells. Although the Chl *b* content in the transformant was 10.6% of total Chl at the early growth phase, the content drastically decreased to 1.4% at the late growth phase (Satoh et al. 2001). Furthermore, the decrease of PS I trimer formation tended to related to the increase of Chl *b* content in the PS I complexes (Satoh and Tanaka 2002). Considering these results, artificially introduced Chl *b* seemed to be rejected by *Synechocystis*. This tendency gave rise to the idea that *Synechocystis* may have lost the tolerance for Chl *b* during evolution if the ancestral cyanobacterium contained other Chl species such as Chl *b*. Therefore, the cyanobacterium with primordial properties is preferable to evaluate properly

the effect of artificially introduced Chl *b* in cyanobacteria that possess no Chl *b*.

Gloeobacter violaceus PCC 7421 is a unicellular cyanobacterium isolated in 1974 (Rippka et al. 1974). *G. violaceus* is the only known species that lacks thylakoid membrane among oxygenic photosynthetic organisms. Molecular phylogenetic analyses indicate that *G. violaceus* is an early-branching cyanobacterium within cyanobacterial clade (Nelissen et al. 1995). Therefore, *G. violaceus* has attracted attention as the model organism of ancestral cyanobacterium. *G. violaceus* has both Chl *a* as the only Chl species and phycobilins in the unique-shaped phycobilisome as antenna pigments (Koyama et al. 2006, Gutiérrez-Cirlos1 et al. 2006). Although the complete genome of *G. violaceus* was sequenced in 2003 (Nakamura et al. 2003), molecular genetic analysis had not been reported for a long time. Recently, I established the transformation system for *G. violaceus* and succeeded in expressing luciferase gene as a reporter gene in *G. violaceus* using expression vectors derived from a broad-host-range plasmid (Araki et al. 2013). Accordingly, I expected that the transformant of *G. violaceus* containing Chl *b* would be the model organism to examine the flexibility of photosystem for Chl species in the ancestral cyanobacterium.

In the present study, I introduced the *CAO* gene derived from prochlorophyte into *G. violaceus*, and the accumulation of Chl *b* in the transformant was analyzed. Furthermore, energy transfer from Chl *b* to Chl *a* in the PS I complexes isolated from the transformant was detected by spectroscopic measurements.

3-2. Materials and Methods

3-2-1. Culture of G. violaceus

G. violaceus was grown in BG11 medium (Allen 1968) under the photoautotrophic conditions ($10 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 25°C, and air was supplied via an air filter (Millex-FG, Millipore, Massachusetts, USA). For transformants, $10 \mu\text{g ml}^{-1}$ streptomycin (Sm) was added

to the medium. Although *G. violaceus* was reported to tend to aggregate during cell growth (Rippka et al. 1974), almost all cells in our laboratory culture do not form aggregate under the above conditions.

3-2-2. Introduction of the CAO gene into *G. violaceus* by conjugation

I amplified the promoter region (185 bp) of *Synechocystis psbA2* gene (Jansson et al. 1998) by PCR using primers, SypsbA2Fw and SypsbA2Rv (Table 2), and the PCR product that contained *KpnI* site, *NdeI* and *KpnI* sites at respective ends was cloned into a cloning vector (pZER0-2, Invitrogen, California, USA). After confirmation of the sequence of the cloned PCR product, the promoter region of *Synechocystis psbA2* gene was excised from the vector by digestion with *KpnI* and *NdeI*. The promoter region of the expression vector with *PhCAO* gene, pKUT1321-*PhCAO* (Tsuchiya et al. 2012B), was replaced by the promoter region of *Synechocystis psbA2* gene to yield pKUT1221-*PhCAO* (Fig. 18). The pKUT1221-*PhCAO* was introduced into *G. violaceus* by diparental mating as described by Araki et al. (Araki et al. 2013). Total DNA was prepared from the transformant cells using hexadecyl-trimethyl-ammonium bromide (Araki et al. 2013, Wilson 1997).

3-2-3. PCR

I confirmed successful introduction of the plasmid into *G. violaceus* by PCR. The *aadA* gene was amplified using the Table 2 primer SmFw and SmRv, the *PhCAO* gene was amplified using the Table 2 primer PhCAOFw and PhCAORv. The enzyme of PCR used *Ex Taq* Hot Start Version (Takara, Shiga, Japan). The equipment for a reaction used thermal cycler, and reaction conditions are shown in Table 3.

3-2-4. Pigment analysis by HPLC

Pigment composition of cells and PS I complexes was analyzed by HPLC as described by Tsuchiya et al. (Tsuchiya et al. 2012A, Tsuchiya et al. 2012B) based on Zapata et al. (Zapata et al. 2000). The pigments extracted with cold methanol from cells or PS I complexes were immediately analyzed by HPLC (Prominence series, Shimadzu, Kyoto, Japan) with a Symmetry C8 column (150 × 1.0 mm, 3.5 µm particle size; Waters, Massachusetts, USA). The elution profile was monitored with a photodiode array detector (SPD-M20A, Shimadzu).

3-2-5. Measurement of growth rate

Measurement of growth rate was used counting chamber (Sunlead Glass Corp, Saitama, Japan). Cells which exist in 0.2 mm × 0.2 mm were measured, and the number of cells per one ml was calculated.

3-2-6. Isolation of PS I complexes

PS I complexes were purified by the procedures described by Inoue et al. (Inoue et al. 2004) based on Sun et al. (Sun et al. 1998) with slight modifications. Cells were harvested by centrifugation (3,800 × g, 5 min, 20°C) and suspended in SMN buffer (0.4 M sucrose, 10 mM NaCl, 50 mM 3-morpholinopropanesulfonic acid (MOPS) (pH 7.0)) containing 0.2 mM phenylmethylsulfonyl fluoride and 5 mM benzamidine. The cells were broken with a bead beater. The membrane fraction was recovered by centrifugation (50,000 × g, 30 min, 4°C) after removal of beads and unbroken cells. This fraction was washed with the SMN buffer containing 5% Sucrose, and CaCl₂ was added at the final concentration of 1 mM. PS I complexes were solubilized with *n*-dodecyl β-D-maltoside (final concentration of 1%) at 4°C for 15 min in the dark. After centrifugation (50,000 × g, 20 min, 4°C), the supernatant was layered on a linear sucrose density-gradient (10–30% sucrose, 0.05% *n*-dodecyl β-D-maltoside, 2 mM NaCl, 10 mM MOPS-NaOH (pH 7.0)) and centrifuged (160,000 × g,

16 h, 4°C). The green band corresponding to the PS I trimer was collected and further purified by anion-exchange column chromatography (UNO Q1, Bio-Rad, California, USA) according to the procedures described by Tomo et al. (Tomo et al. 2008B). The purified PS I fraction was concentrated using Amicon Ultra Centrifugal Filters (0.5 ml 100K, EMD Millipore, Massachusetts, USA), and the buffer was replaced by SMN buffer containing 0.04% *n*-dodecyl β -D-maltoside.

3-2-7. Steady-state absorption and fluorescence spectroscopy

Absorption spectra of cells at 80 K were measured by Hitachi 557 spectrophotometer (Hitachi, Tokyo, Japan) which was equipped with the commercially available Dewar bottle (Mimuro et al. 1999, Mimuro et al. 2010). Low-temperature absorption and fluorescence spectra were measured basically as reported previously (Tomo et al. 2008A). For the measurement of fluorescence spectra at 77 K, Hitachi F-4500 fluorescence spectrophotometer (Hitachi) which was equipped with a custom made Dewar system (Mimuro et al. 1999) was used. The density of cells was adjusted to 3 $\mu\text{g Chl } a \text{ ml}^{-1}$ by BG11 medium for the low-temperature fluorescence measurement. The concentration of PS I complexes was adjusted to 2 $\mu\text{g total Chl ml}^{-1}$ for the low-temperature absorption measurement.

3-2-8. SDS-PAGE

SDS-PAGE was performed according to the standard procedure (Ikeuchi and Inoue 1988) using a 16–22% separating gel with a 6% stacking gel that contained 7.5 M urea. PS I of 4 $\mu\text{g Chl}^{-1}$ was used for electrophoresis. The gels were stained with CBB after electrophoresis.

3-2-9. Time-resolved fluorescence spectroscopy

Time-resolved fluorescence spectra in picosecond to nanosecond region were measured with a time-correlated single-photon counting system at 77 K (Tomo et al. 2008B). The excitation wavelength was 425 nm, at which all pigments (Chl *a*, Chl *b* and carotenoids) were simultaneously excited. The time window of measurement was 10 ns with an interval of 2.4 ps/channel. To construct the time-resolved fluorescence spectra (TRFS), fluorescence rise and decay curves were measured in the Chl *b* to Chl *a* fluorescence region (650–750 nm) with an interval of 1 nm/channel. Fluorescence rise and decay curves in femtosecond to picosecond region were measured by the fluorescence up-conversion method with an interval of 33.3 fs/channel at 293 K (Akimoto et al. 2005). The excitation wavelength was 425 nm, whereas the observed wavelength was 660 nm (the Chl *a* fluorescence in the control PS I, whereas the Chl *a* and Chl *b* fluorescences in the Chl *b*-containing PS I), 680 nm (the Chl *a* fluorescence in both samples), and 690 nm (the Chl *a* fluorescence in both samples). In all the time-resolved measurements, I observed the polarization component of the fluorescence signals at the magic angle to the excitation laser pulse.

3-3. Results

3-3-1. Introduction of the CAO gene into *G. violaceus*

I had already subcloned the *CAO* gene originated from a prochlorophyte, *Prochlorothrix hollandica* (*PhCAO*) (Nagata et al. 2004) into an expression vector to yield pKUT1321-*PhCAO* (Tsuchiya et al. 2012B). The promoter region of the pKUT1321-*PhCAO* was replaced by *psbA2* promoter (Jansson et al. 1998) derived from *Synechocystis*. The resultant vector, pKUT1221-*PhCAO* was introduced into *G. violaceus* by conjugal gene transfer according to the method of Araki et al. (Araki et al. 2013). Sm resistant exconjugants were obtained, and total DNA prepared from an exconjugant was used as template of PCR to confirm the success of transformation. Total DNA prepared from wild type and from

pKUT1121 strain (Araki. et al. 2013) that harbors the expression vector (pKUT1121) without *PhCAO* gene were used for control. The Sm resistance gene, *aadA* gene (Fig. 19A), was amplified in total DNA prepared from the pKUT1121 strain and from the Sm-resistant exconjugant by PCR (Fig. 19B, lanes 2 and 3), while no band was amplified in total DNA prepared from wild type (Fig. 19B, lane 1). Furthermore, *PhCAO* was also amplified (Fig. 19A) only in the total DNA from the Sm-resistant exconjugant (Fig. 19C, lane 3). Hence, it was demonstrated that *aadA* gene and *PhCAO* gene existed in the Sm-resistant exconjugant. Furthermore, I transformed *E. coli* with the total DNA from the Sm-resistant exconjugant, and the plasmids prepared from *E. coli* transformants and the original pKUT1221-*PhCAO* were digested with the *Xho* I, *Mlu* I and *Nde*I and *Xho*I. In agarose gel electrophoresis (Fig. 20), the restriction patterns of the prepared plasmids (Fig. 20, lanes 2, 4 and 6) were identical to that of the original pKUT1221-*PhCAO* (Fig. 20, lanes 1, 3 and 5). Therefore, I concluded that the Sm-resistant exconjugant harbors pKUT1221-*PhCAO* as a plasmid.

3-3-2. Pigment composition of *G. violaceus* transformants

I analyzed the pigment composition of *G. violaceus* (pKUT1221-*PhCAO*) (hereafter referred to as pKUT1221-*PhCAO* strain) by high performance liquid chromatography (HPLC) using the method of Tsuchiya et al. (Tsuchiya et al 2012A, Tsuchiya et al. 2012B) (Fig. 21). The pigment composition of the pKUT1121 strain was analyzed as control. Figure 21A shows HPLC chromatogram of methanol extract of each strain monitored at 462 nm that is the absorption maximum of Chl *b*. Like wild type (Tsuchiya et al. 2005), (2S,2'S)-oscillol 2,2'-di(α -L-fucoside) (peak 1), echinenone (peak 2), Chl *a* (peak 3) and β -carotene (peak 4) were detected in both the pKUT1121 strain and the pKUT1221-*PhCAO* strain. Additionally, a novel peak (peak 5) was detected only in the pKUT1221-*PhCAO* strain with the retention time at 41.4 min. The retention time and the absorption spectrum (Fig. 21B) of the peak 5

were identical to those of Chl *b*, indicating that the pKUT1221-*PhCAO* strain accumulated Chl *b* in addition to Chl *a*. The Chl *b* accumulated at approximately 11% of total Chl in the cells. Furthermore, the carotenoid content relative to total Chl seemed to be high in the pKUT1221-*PhCAO* strain. In particular, the molar ratio of (2S,2'S)-oscilloxanthin to total Chl was approximately 1.8 times higher in the pKUT1221-*PhCAO* strain than that in the pKUT1121 strain.

3-3-3. Changes in the Chl b content during cell growth in the pKUT1221-PhCAO strain

Growth of each transformant was monitored by cells number. Comparing growth curves, I found that the pKUT1221-*PhCAO* strain grew slightly slower than the pKUT1121 strain (Fig. 22). The mean generation time was calculated based on each growth curve, 78 h for the pKUT1121 strain, and 90 h for the pKUT1221-*PhCAO* strain. I measured Chl content of the pKUT1221-*PhCAO* strain during cell growth (Fig. 23). Total Chl per milliliter culture increased during cell growth like the increase of cell number (Fig. 22). The molar ratio of Chl *b* to total Chl was approximately 11% and kept at a constant level. This result means that Chl *b* is continuously synthesized independent of growth phase in the pKUT1221-*PhCAO* strain.

3-3-4. Low-temperature absorption spectra and fluorescence spectra of G. violaceus transformant cells

Since pigment composition of the pKUT1221-*PhCAO* strain differed from that of the pKUT1121 strain, the absorption spectra of transformant cells were measured at 80 K (Fig. 24). The absorption corresponding to Chl *a*, allophycocyanin and phycocyanin in the pKUT1221-*PhCAO* strain were relatively lower than the pKUT1121 strain. Conversely, the difference absorption spectrum clearly showed the absorption maximum at 458 nm, which might be the absorption maximum of the Soret band of Chl *b* in the pKUT1221-*PhCAO*

strain. Then, steady-state fluorescence spectra of the transformant cells were measured at 77 K (Fig. 25). I selected three excitation wavelengths, 440 nm (Fig. 25A) mainly for Chl *a*, 560 nm (Fig. 25B) mainly for phycoerythrin, 458 nm (Fig. 25C) which was a peak wavelength of low-temperature difference absorption spectrum (Fig. 24). Upon excitation at 440 nm (Fig. 25A), no additional peak was detected in the pKUT1221-*PhCAO* strain, and the profile of the spectrum was similar to that of the pKUT1121 strain. The maximum wavelength of the pKUT1221-*PhCAO* strain was 684 nm, which was 1 nm shorter than that of the pKUT1121 strain (685 nm). Upon excitation at 560 nm and 458 nm, fluorescence intensity at the maximum wavelength of phycocyanin (644.4 nm) of the pKUT1221-*PhCAO* strain was slightly higher than that of the pKUT1121 strain (Figs. 25B and 25C). Only upon excitation at 458 nm (Fig. 25C), difference between two spectra was observed in the region longer than 700 nm. In this wavelength region, fluorescence intensity of the pKUT1221-*PhCAO* strain was slightly lower than that of the pKUT1121 strain.

3-3-5. Isolation of PS I complexes from the pKUT1221-PhCAO strain

For further analysis of the pKUT1221-*PhCAO* strain, PS I complexes were isolated. The solubilized cell membrane was layered on a linear sucrose density-gradient (10–30%), and the gradient was centrifuged to separate PS I complexes. As to the pKUT1121 strain, a major band corresponding to trimeric PS I and a faint band corresponding to the mixture of monomeric PS I and PS II were observed (Fig. 26). This sedimentation pattern was similar to that of wild type as reported previously (Inoue et al. 2004). For the pKUT1221-*PhCAO* strain (Fig. 26, lane 2), sedimentation pattern was the same as that of the pKUT1121 strain (Fig. 26, lane 1).

The trimeric PS I fraction separated by the sucrose density-gradient centrifugation was further purified by anion-exchange column chromatography. Then, pigment composition of

the isolated PS I complexes was analyzed by HPLC (Fig. 27). The number of pigment molecules in the PS I complexes isolated from the pKUT1121 strain (control PS I) was 108 Chl *a* molecules and 23 β -carotene molecules per one Chl *a'* molecule. In contrast, the number of pigment molecules in the PS I complexes isolated from the pKUT1221-*PhCAO* strain (Chl *b*-containing PS I) was 128 Chl *a* molecules, 14 Chl *b* molecules and 32 β -carotene molecules per one Chl *a'* molecule. Although each value for the Chl *b*-containing PS I was higher than that for control PS I, the molar Chl *b* ratio to total Chl was approximately 9.9%. This ratio was nearly equal to the ratio in cells. The polypeptide composition of the isolated PS I complexes was examined by SDS-PAGE (Fig. 28). The peptide pattern of the Chl *b*-containing PS I (Fig. 28, lane 2) was similar to that of control PS I (Fig. 28, lane 1); the principal bands were clearly detected in both samples with almost identical stoichiometry. These results demonstrated that the replacement of a part of Chl *a* molecules by Chl *b* molecules in PS I complexes does not affect the trimer formation of PS I complexes in *G. violaceus*.

3-3-6. Spectroscopic analysis of the *G. violaceus* PS I complexes that contain Chl *b*

I measured the low-temperature absorption spectra of the PS I complexes with the same total Chl concentration (Fig. 29). The difference absorption spectrum between two PS I complexes showed the presence of the positive peaks at 466 nm and 654 nm and negative peaks at 685 nm, 442 nm and 418 nm. The positive peaks were similar to the profile of the absorption spectrum of Chl *b*. Therefore, the replacement of a part of Chl *a* molecules by Chl *b* molecules in the Chl *b*-containing PS I was also confirmed by the low-temperature absorption spectra.

Fluorescence rise and decay curves in femtosecond to picosecond region were measured to examine whether Chl *b* inserted in PS I complexes have contributed to the energy transfer

to Chl *a* (Fig. 30). As a result of global analysis (solid lines in Fig. 30), three time constants and their amplitudes were obtained (Table 7). The lifetime values of the first component were almost the same in two PS I complexes (100–110 fs). The first component was resolved as a decay component (a positive amplitude) at 660 nm and as a rise component (a negative amplitude) at 680 nm and 690 nm, independent of the existence of Chl *b*. Therefore, I assigned this component due to an internal conversion from Q_x to Q_y in Chl *a* (Akimoto et al. 1999). The lifetime value of the second component in the control PS I was analyzed to be 1.12 ps. Contribution of the 1.12-ps component was small ($\leq 5\%$), but necessary for the best fit. This component was suggested to be the vibrational relaxation in Q_y (Akimoto et al. 1999) and/or the energy transfer between Chl *a* (Akimoto et al. 2005). In contrast, the lifetime value of the second component in the Chl *b*-containing PS I was analyzed to be 510 fs, and the amplitude of the 510-fs component was five times larger than that of the second component in the control PS I at 660 nm (Table 7), indicating contribution of the Chl *b* fluorescence to the 510-fs component at 660 nm in the Chl *b*-containing PS I. Additionally, the 510-fs component exhibited negative amplitudes in the Chl *a* fluorescence region (680 nm and 690 nm). Therefore, the 510-fs component was suggested to be mainly coming from the energy transfer from Chl *b* to Chl *a*. The fluorescence decay of the third component was observed with almost the same lifetime in two PS I complexes (13.1–13.6 ps), and this was assigned due to thermal equilibrium among Chl pigment pools (Akimoto et al. 2005). Then, I further measured TRFS in picosecond to nanosecond region at 77 K for the two PS I complexes (Fig. 31). There were no significant differences between two sets of TRFS. Therefore, it was concluded that Chl *b* molecules in the Chl *b*-containing PS I did not affected the energy transfer among the Chl *a* molecules in the picosecond to nanosecond region.

3-4. Discussion

As to the *CAO*-introduced *Synechocystis*, the drastic decrease in the molar ratio of Chl *b* in the cells from 10.6% to 1.4% during cell growth was reported (Sato et al. 2001). However, the *PhCAO*-introduced *G. violaceus* accumulated Chl *b* approximately 11% of total Chl independent of growth phase (Fig. 23). This inconsistency can be explained as follows. *Synechocystis* has unknown mechanism to exclude artificially produced Chl *b* molecules, and *G. violaceus* does not possess the mechanism, which may include the inhibition of Chl *b* biosynthesis. The major difference of *G. violaceus* from *Synechocystis* is the lack of thylakoid membrane. However, there is no evidence that suggests the relationship between the existence of thylakoid membranes and the tolerance for Chl *b*. Xu et al. (Xu et al. 2001) reported that Chl *b* accumulated in cells at approximately 60% of total Chl when both *lhcb* gene encoding light-harvesting chlorophyll-protein complex II (LHC II) and *Arabidopsis CAO* cDNA were introduced into *Synechocystis* mutant that lost both the PS I and *chlL* gene encoding a subunit of the light-independent protochlorophyllide *a* reductase. However, without co-expression of *lhcb* gene, Chl *b* amount decreased to approximately 6% (Xu et al. 2001). Since these mutants cannot grow under the photoautotrophic conditions because of the lack of PS I, above result clearly indicated that capability of photosynthesis is not the limiting factor for the restriction of artificially produced Chl *b* molecules in the transformant. Furthermore, Chl *b* content per cell may be low in the above mutant that contains both *CAO* gene and *lhcb* gene because Chl content of PS I-less mutant is much lower than wild type. Sato et al. (Sato et al. 2001) also discussed the importance of the coexistence of the Chl *b*-binding antenna proteins such as Pcb and LHC II for Chl *b* accumulation. However, *G. violaceus* have neither Pcb nor LHC II, and no genes encoding them found in the genome. Therefore, further studies will be needed to prove the relationship between the accumulation of Chl *b* and the existence of antenna protein.

In the *CAO*-introduced *Synechocystis*, the formation of PS I trimer was inhibited correlated with the accumulation of Chl *b* (Sato and Tanaka 2002). In contrast, the *PhCAO*-introduced *G. violaceus*, no similar inhibition was observed (Fig. 26). Unlike other cyanobacteria including *Synechocystis*, almost all PS I complexes form trimer during sucrose density-gradient centrifugation in the case of *G. violaceus* (Inoue et al. 2004). Mangels et al. (Mangels et al. 2002) demonstrated that the PS I complexes isolated from *G. violaceus* showed a novel T-shaped complex by electron microscopy. In addition, I found the C-terminal extension that was similar to the peptidoglycan-binding domain in *G. violaceus* PsaB (Inoue et al. 2004). Taken these findings together, three C-terminal extensions of PsaB from trimer are expected to form a bundle at the center of trimer. Although the bundle may tighten the PS I complexes in the trimer, no experimental evidence for the function of the domain has been reported to date. Because the disruption of the *psaL* gene required for the trimer formation of PS I complexes did not affect the growth and photosynthetic characteristics in *Synechocystis* (Chitnis et al. 1993), the inhibition of the trimer formation might not be important for *Synechocystis*. Therefore, I concluded that the trimer formation of PS I complexes is not directly related to the tolerance for artificially produced Chl *b* molecules.

By pigment analysis of PS I complexes, I found the apparent increase of pigments in the Chl *b*-containing PS I compared to the control PS I. For the pigment content of PS I, the three-dimensional structure of cyanobacterial PS I revealed the presence of 96 Chl molecules, 22 carotenoid molecules and 1 Chl *a'* molecule (Jordan et al. 2001). Furthermore, approximately 100 Chl molecules were found in the core complex of plant PS I by the three-dimensional structure, and the position of vast majority of the Chl molecules was almost identical to that in the cyanobacterial PS I (Amunts et al. 2007). For the control PS I, the pigment composition (108 Chl *a* molecules and 23 β -carotene molecules per one Chl *a'*

molecule) is similar to that determined from the three-dimensional structures of both cyanobacterium and plant. Therefore, the pigment composition of the Chl *b*-containing PS I (128 Chl *a* molecules, 14 Chl *b* molecules and 32 β -carotene molecules per one Chl *a'* molecule) is quite abnormal. I estimated the increasing rate of each pigment to be 1.31 and 1.39 for total Chl and β -carotene, respectively. Since these rates are similar to each other, I hypothesized that not all PS I complexes contain Chl *a'* molecule. If 30% of the Chl *b*-containing PS I have no Chl *a'* molecule, pigment composition is estimated to be 90 Chl *a* molecules, 10 Chl *b* molecules and 22 β -carotene molecules. Although these values are reasonable, further studies must be needed to prove this hypothesis.

Regarding steady-state fluorescence spectra, no significant difference between two strains was observed when cells were excited at 440 nm (Fig. 25A). This result means that the energy transfer between Chl *a* molecules was not changed, and this explanation is consistent with TRFS (Fig. 31). The fluorescence intensity at the maximum wavelength of phycocyanin was increased in the pKUT1221-*PhCAO* strain upon excitation at 560 nm and 458 nm (Figs. 25B and 25C). This may reflect the different phycobiliprotein composition of phycobilisome indicated by the low-temperature absorption spectra of cells (Fig. 24). Only upon excitation at 458 nm, decrease in the fluorescence intensity in the pKUT1221-*PhCAO* strain was observed in the region longer than 700 nm (Fig. 25C). This result can be explained by the absence of Chl *a* molecules with lower energy level near the Chl *b* molecules in the cells.

Because the TRFS in femtosecond region of PS I containing Chl *b* prepared from the *CAO*-introduced *Synechocystis* has not been measured, energy transfer from Chl *b* molecule to Chl *a* molecule was not clear. Since the *PhCAO*-introduced *G. violaceus* was suitable for isolating the PS I complexes with high Chl *b* content, I used the pKUT1221-*PhCAO* strain as a source of the Chl *b*-containing PS I. The TRFS of the Chl *b*-containing PS I at the femtosecond revealed the energy transfer from Chl *b* molecule to Chl *a* molecule (Table 7).

The time constant (510 fs) was almost the same as that of the energy transfer from Chl *b* molecule to Chl *a* molecule in LHC II (500 ± 200 fs) (Eads et al. 1989, Visser et al. 1996). This result shows the direct energy transfer from Chl *b* molecule to Chl *a* molecule in the Chl *b*-containing PS I. Furthermore, the fluorescence (650 nm) derived from Chl *b* was not detected by measurement in TRFS at picosecond, and significant differences from the control PS I were not found. These results suggested that free Chl *b* did not exist, and all the Chl *b* molecules in PS I effectively transfer energy to Chl *a* molecule.

In the present study, I demonstrated that a part of Chl *a* molecules in the PS I complexes were replaced by Chl *b* molecules in the *PhCAO*-introduced *G. violaceus*, and the accumulation of Chl *b* did not inhibit the trimer formation of PS I complexes. This means that *G. violaceus* is more tolerant to artificially produced Chl *b* than *Synechocystis*. Furthermore, effective energy transfer from artificially produced Chl *b* to Chl *a* in PS I complexes was directly measured for the first time. The replacement of Chl *a* molecules by Chl *b* molecules in PS I complexes without functional defect indicates that the coexistence of the Chl *b*-binding antenna protein, Pcb is not essential for the use of Chl *b* in *G. violaceus*. Although my results cannot ensure the presence of the ancestral cyanobacterium that contained both phycobilin and Chl *b* as antenna pigments, the flexibility of Chl composition found in the thylakoid-lacking cyanobacterium, *G. violaceus* will provide new insight into the evolution of Chls and photosystems. This molecular biological study of *G. violaceus* revealed its unique properties that might be originated from the primordial cyanobacteria and were differentiated from the other cyanobacteria. Therefore, *G. violaceus* appears to be the important organisms to understand the evolution and diversity of the oxygenic photosynthetic organisms including cyanobacteria.

Chapter 4

Summary and perspectives

In this study, I showed the following two points.

(1) In Chapter 2, I discussed the development of the transformation system and establishment of reporter system. Transformation of *G. violaceus* was reported by Guo and Xu (2004). However, *G. violaceus* of our laboratory was not able to survive under the conditions of Guo and Xu (2004) (Fig. 10). I can be cultivating on the same conditions as Rippka et al. (1974). Hence, I suggested that the property of *G. violaceus* used in Guo and Xu (2004) was different from our laboratory *G. violaceus* by unknown reason. Therefore, I developed transformation system for *G. violaceus* applying the conditions of our laboratory. Since wild type *G. violaceus* showed susceptibility to Sm, I transformed *G. violaceus* using the expression vector (pKUT1121) (Tsuchiya et al. 2012B) derived from a broad-host-range plasmid, RSF1010, by conjugation. Transformation efficiency of the obtained exconjugants was 7.7 ~ 26 times higher than that reported by Guo and Xu (2004), indicating that I succeeded in developing a transformation system more efficient than the previously described system Guo and Xu (2004). I transformed *E. coli* XL1-Blue MRF' (*mcrA*⁻) and *E. coli* DH5 α (*mcrA*⁺) with the total DNA prepared from *G. violaceus* transformant harboring pKUT1121, and obtained Sm-resistant transformants from both *E. coli* strains. The plasmids prepared from the *E. coli* transformants and original pKUT1121 were digested with restriction enzymes. As a result, these plasmids were identical to that of the original pKUT1121 judging from the pattern of the restriction fragments (Fig. 13). This result was in agreement with genomic analysis. Therefore, these results indicated that pKUT1121 introduced into *G. violaceus* was maintained as plasmid without modification in *G. violaceus*. Furthermore, I introduced the *luc* gene derived from firefly into multiple cloning site of pKUT1121 to yield pKUT1121-*luc*. In *G. violaceus* transformed with pKUT1121-*luc* (pKUT1121-*luc* strain), luciferase was accumulated, and the expression level would be enough for the metabolic engineering of *G. violaceus* (Fig. 16). The following two points became clear from luciferase

assay (Table 6); no background luminescence (without luciferase) was detected from *G. violaceus*, luciferase activity was detected only in pKUT1121-*luc* strain. Therefore, the *luc* gene was suggested to be an effective reporter gene in *G. violaceus*, and the developed transformation system was useful for metabolic engineering of *G. violaceus*.

In conclusion, I succeeded in introducing the expression vectors derived from the broad-host-range plasmid into *G. violaceus* by conjugal gene transfer. Furthermore, the luciferase expressed in *G. violaceus* functioned as a reporter.

(2) In Chapter 3, since Tomitani et al (1999) suggested that the common ancestor of cyanobacteria and chloroplasts had Chl *b* and phycobilins in addition to Chl *a*, I produced the *G. violaceus* transformant which artificially accumulated Chl *b* in addition to Chl *a* using the transformation system developed in Chapter 2. I discussed about the effect of the accumulated Chl *b* on photosynthesis in the transformant. From the pigment analysis of the transformant using HPLC, Chl *b* was contained approximately 11% of total Chl, and the ratio was constant independent of growth phase (Fig. 23). In contrast, in the *Arabidopsis* CAO-introduced *Synechocystis*, Chl *b* content drastically decreased from 10.6% to 1.4% during cell growth (Sato et al. 2001). Although *Synechocystis* might have unknown mechanism to exclude artificially produced Chl *b* molecules, the cause of this difference between two species is unclear. Further studies will be needed to clarify the reason of this difference.

Tomitani et al (1999) also suggested that Chl *b* in the common ancestor bound to Pcb or LHC II which are Chl *b*-binding proteins. *G. violaceus* does not have Pcb and LHC II, and no gene encoding the proteins were found in the genome sequence. However, since PhCAO-introduced *G. violaceus* consistently contained Chl *b* approximately 11% of total Chl without defect of photosynthetic reaction in PS I, presence of Chl *b*-binding protein may not

be indispensable. TRFS showed that Chl *b* molecule in the PS I prepared from the *PhCAO*-introduced *G. violaceus* was transferred energy to Chl *a* molecule directly. The absence of free Chl *b* molecule in the PS I indicating the functional replacement of several Chl *a* molecules with Chl *b* molecules (Figs. 30 and 31 and Table 8).

It was concluded that I produced transformants which contained Chl *b* in addition to Chl *a*. The transformant constantly accumulated approximately 11% Chl *b* of total Chl. In PS I of this transformant, a part of Chl *a* molecules are replaced by Chl *b* molecules. This was the first time to demonstrate the direct energy transfer from Chl *b* to Chl *a* in PS I which artificially accumulated Chl *b* by TRFS.

The results of this research suggested that molecular genetic study of *G. violaceus* provide new knowledge about evolution of Chls and photosynthesis.

In this study, I aimed to develop the experimental system for molecular genetics study for *G. violaceus*. The successful alteration of the Chl biosynthetic pathway by introducing *CAO* gene indicates that other metabolic pathway in *G. violaceus* can be altered using the system developed in this study. Such experiments will provide new knowledge and results that cannot be obtained by the study of other oxygenic photosynthetic organisms. Although I succeeded in introducing foreign genes into *G. violaceus* (Fig. 32A), development of other systems for various molecular genetic analyses such as gene targeting (Fig. 32B) and comprehensive gene disruption (Fig. 32C). The development of these experimental systems requires further examination, however, I consider that the systems can be developed near future, because the development of transformation system is the most difficult barrier for the systems. Therefore, the transformation system developed by this study is the very important technology as the base for the molecular genetic analysis of *G. violaceus*.

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Table 1 Features of photosynthetic organisms

Domain	Bacteria	Bacteria	Eukaryota	Eukaryota
Popular name	Photosynthetic bacteria	Cyanobacteria	Algae	Plants
Terminal electron donor	H ₂ S, S, organic acids, thiosulfate	H ₂ O	H ₂ O	H ₂ O
Oxygen evolution	No	Yes	Yes	Yes
Number of reaction center	1	2	2	2
Type of chlorophylls	Bacteriochlorophyll	Chlorophyll	Chlorophyll	Chlorophyll

Table 2 Oligonucleotides used in this study

Name	Sequence
SmFw	5'-ATGCGCTCACGCAACTGGTCCAG-3'
SmRv	5'-TTATTTGCCGACTACCTTGGTGATCTC-3'
lucFw	5'-GGGCATATGGAAGACGCCAAAAACAT-3'
lucRv	5'-TTTCTCGAGTTACACGGCGATCTTTCCGC-3'
SypsbA2Fw	5'-GGTACCTACCCATGGAAAAAACGACAATTA-3'
SypsbA2Rv	5'-GGTACCCATATGGTTATAATTCCTTATGTA-3'
PhCAOFw	5'-CATATGAATAATAGCCTCAACGTATCCGC-3'
PhCAORv	5'-CTCGAGTCAGTGCCCCGTGGGAGGAG-3'

Sequences corresponding to the restriction sites introduced are underlined.

Table 3 The cycling conditions of PCR

Reaction	Temperature (°C)	Time (min)	
Denaturation	98	1.5	
Denaturation	98	0.5	} 20 cycles
Annealing	59	0.5	
Extension	72	2.0	
Extension	72	2.0	
Hold	4	∞	

Table 4 Antibiotic susceptibility of *G. violaceus* in BG11 liquid culture medium

Antibiotics	Concentration ($\mu\text{g ml}^{-1}$)			
	1	5	15	50
Ap	R	R	R	R
Cm	R	R	R	S
Em	S	S	S	S
Gm	R	R	R	S
Hyg	R	R	R	R
Km	R	R	R	R
Sm	R	S	S	S
Sp	R	S	S	S
Zeo	R	R	R	R

Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Hyg, hygromycin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Zeo, zeocin.

R, resistant; S, sensitive.

Table 5 Antibiotic susceptibility of *G. violaceus* on BG11 agar medium

Antibiotics	Concentration ($\mu\text{g ml}^{-1}$)			
	1	5	15	50
Em	R	R	R	S
Sm	R	S	S	S
Sp	R	R	R	S

Em, erythromycin; Sm, streptomycin; Sp, spectinomycin.

R, resistant; S, sensitive.

Table 6 Luciferase activity of wild type and *G. violaceus* transformants

Samples	Background (RLU)*	+Luciferin (RLU) *
Wild type	118 ± 16	106 ± 32
pKUT1121 strain	83 ± 5	87 ± 9
pKUT- <i>luc</i> strain	87 ± 11	110,437 ± 10,431

*RLU, Relative luminescence units.

The values represent the averages and standard deviations of triplicate measurements.

Table 7 The summary of lifetime values and their normalized amplitudes of the PS I complexes isolated from *G. violaceus* transformants at room temperature. Positive and negative amplitudes correspond to decay and rise of fluorescence, respectively. The sum of the positive amplitude(s) was normalized to be 100% at each observed wavelength (λ).

λ	Control PS I			Chl <i>b</i> -containing PS I		
	110 fs	1.12 ps	13.6 ps	100 fs	510 fs	13.1 ps
660 nm	74.8%	5.2%	20.0%	45.3%	26.4%	28.3%
680 nm	-55.9%	2.7%	97.3%	-41.0%	-3.3%	100%
690 nm	-93.7%	-3.9%	100%	-79.9%	-8.2%	100%

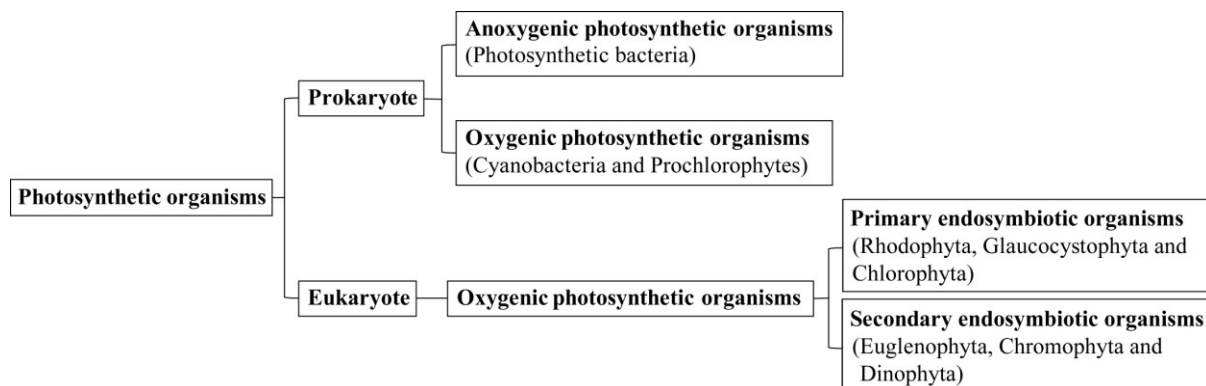


Fig. 1 Classification of photosynthetic organisms

Photosynthetic organisms are classified into prokaryote and eukaryote. Prokaryote is classified into anoxygenic photosynthetic organisms and oxygenic photosynthetic organisms. Anoxygenic photosynthetic organisms are called photosynthetic bacteria, and cyanobacteria including prochlorophytes are the only prokaryotic oxygenic photosynthetic organisms. All eukaryotic photosynthetic organisms are oxygenic phototrophs. Eukaryotic photosynthetic organisms are classified by the number of endosymbiosis.

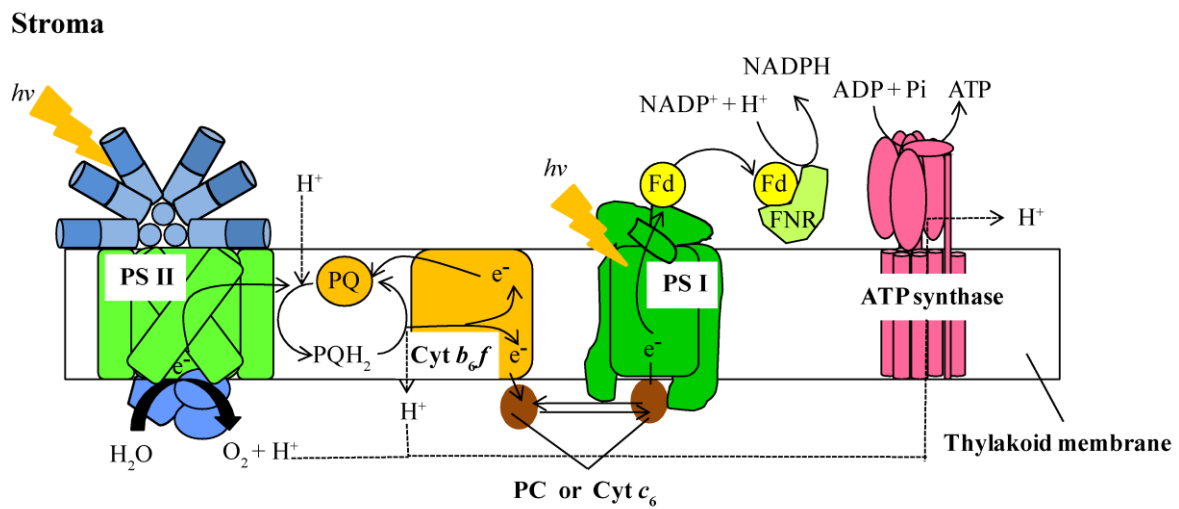


Fig. 2 Electron transfer in the thylakoid membrane of cyanobacteria

$h\nu$, light; PS II, photosystem II; PQ, plastoquinone; PQH_2 , reduced plastoquinone; Cyt b_6f , cytochrome b_6f complex; PC, plastocyanin; Cyt c_6 , cytochrome c_6 ; PS I, photosystem I; Fd, ferredoxin; FNR, ferredoxin:NADP⁺ oxidoreductase.

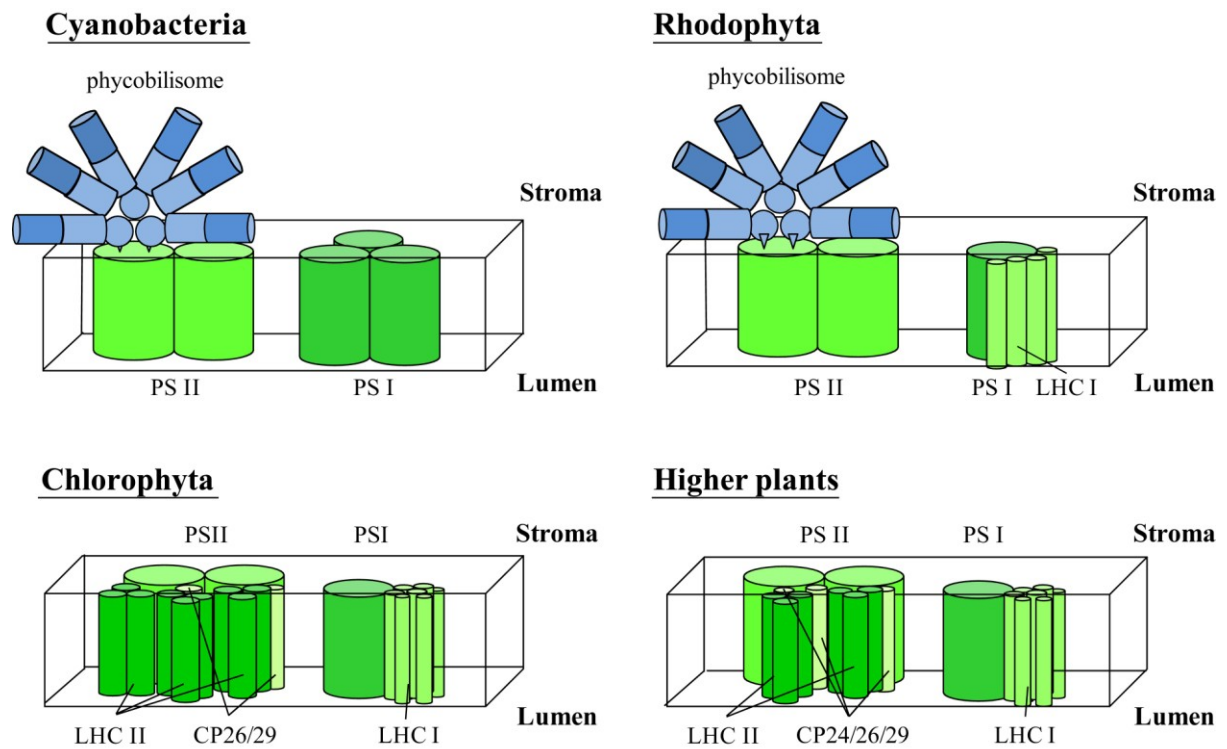


Fig. 3 The antenna model of oxygenic photosynthetic organisms

Cyanobacteria and Rhodophyta have phycobilisome as a light-harvesting antenna of PS II. Rhodophyta also have light harvesting chlorophyll-protein complex I (LHC I) for PS I. Chlorophyta and plants have light harvesting chlorophyll-protein complex II (LHC II) and CP24/26/29 instead of phycobilisome. Additionally, Chlorophyta also have LHC I for PS I.

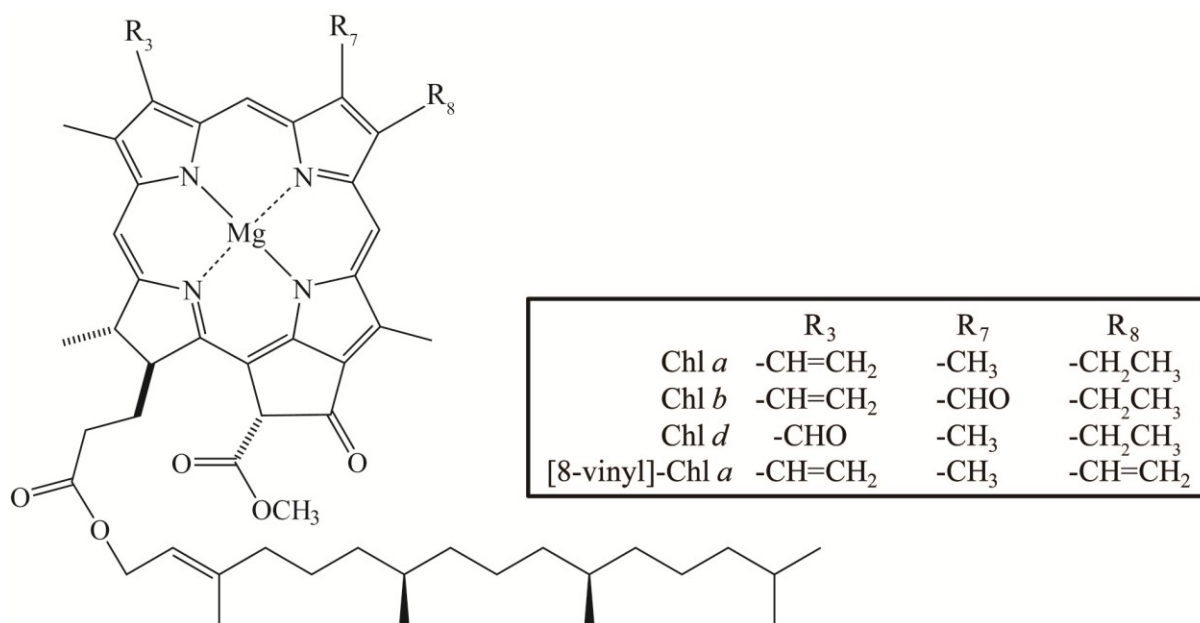


Fig. 4 The chemical structures of chlorophylls related to this study

R₃, R₇ and R₈ of Chl *a* are vinyl group, methyl group and ethyl group, respectively. R₃, R₇ and R₈ of Chl *b* are vinyl group, formyl group and ethyl group, respectively. R₃, R₇ and R₈ of Chl *d* are formyl group, methyl group and ethyl group, respectively. R₃, R₇ and R₈ of [8-vinyl]-Chl *a* are vinyl group, methyl group and vinyl group, respectively.

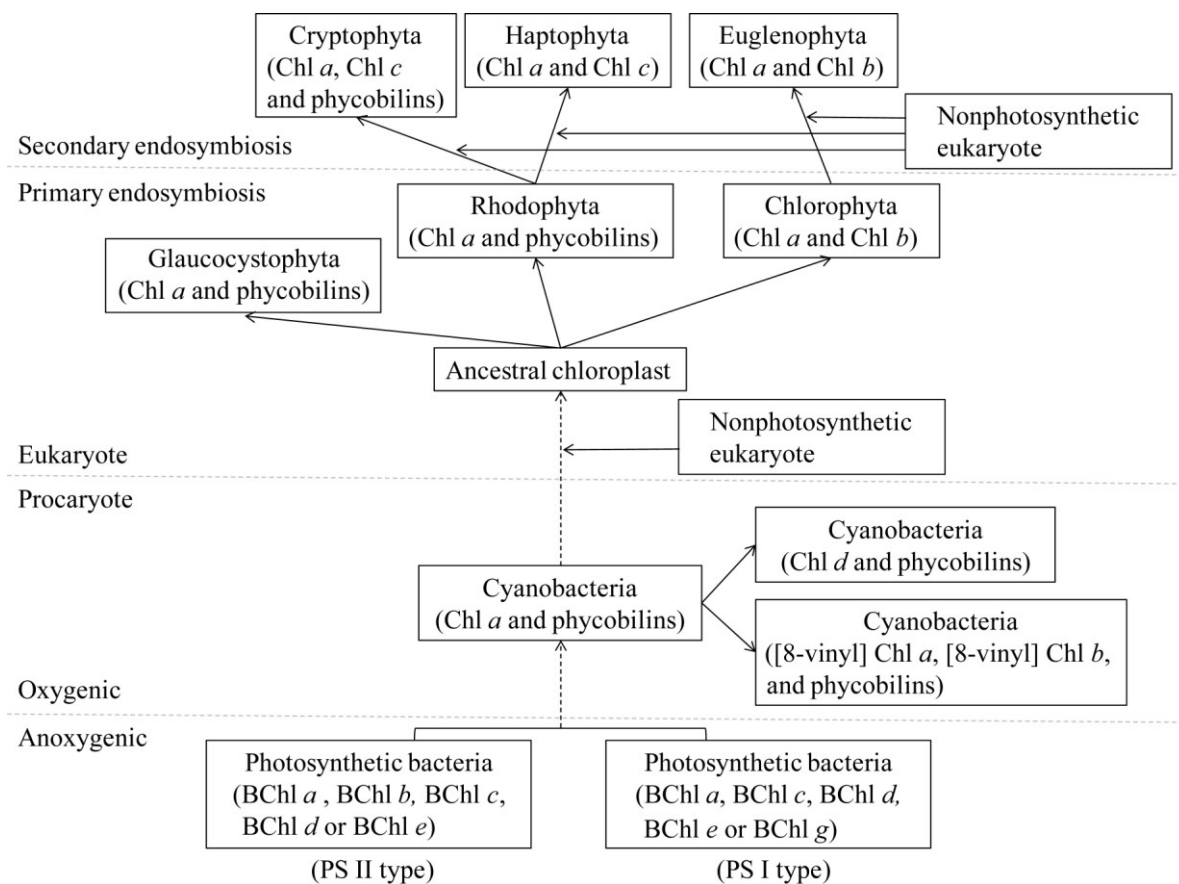


Fig. 5 The hypothetical scheme of the evolution of photosynthetic organisms

Black dashed arrow, relevance has many mysteries; black arrows, relevance has high; gray dashed arrow, the difference from organisms.

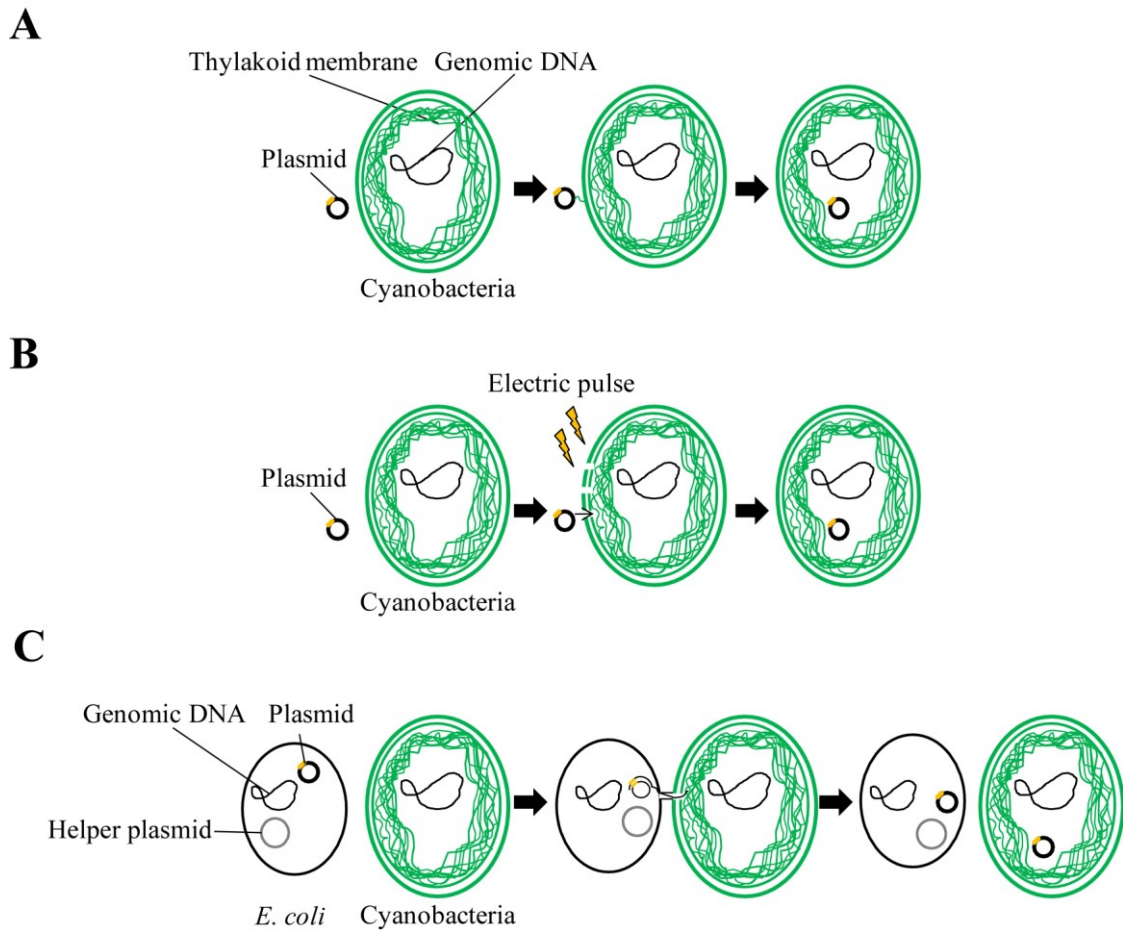


Fig. 6 The schematic view of transformation techniques of cyanobacteria

(A) Natural transformation. (B) Electroporation. (C) Conjugation. Natural transformation (A) is naturally occurring that plasmid is taken into cyanobacteria by cilia of cyanobacteria when plasmid and cyanobacteria are mixed. Electroporation (B) is a mechanical transformation technique that uses an electrical pulse to create temporary pores in cell wall and plasma membrane through which plasmid can pass into cells. Conjugation (C) is a method of the transfer of plasmid between bacterial cells. When conjugation of *E. coli* which contains the helper plasmid and the plasmid and cyanobacteria, plasmid in *E. coli* is transmitted to cyanobacteria.

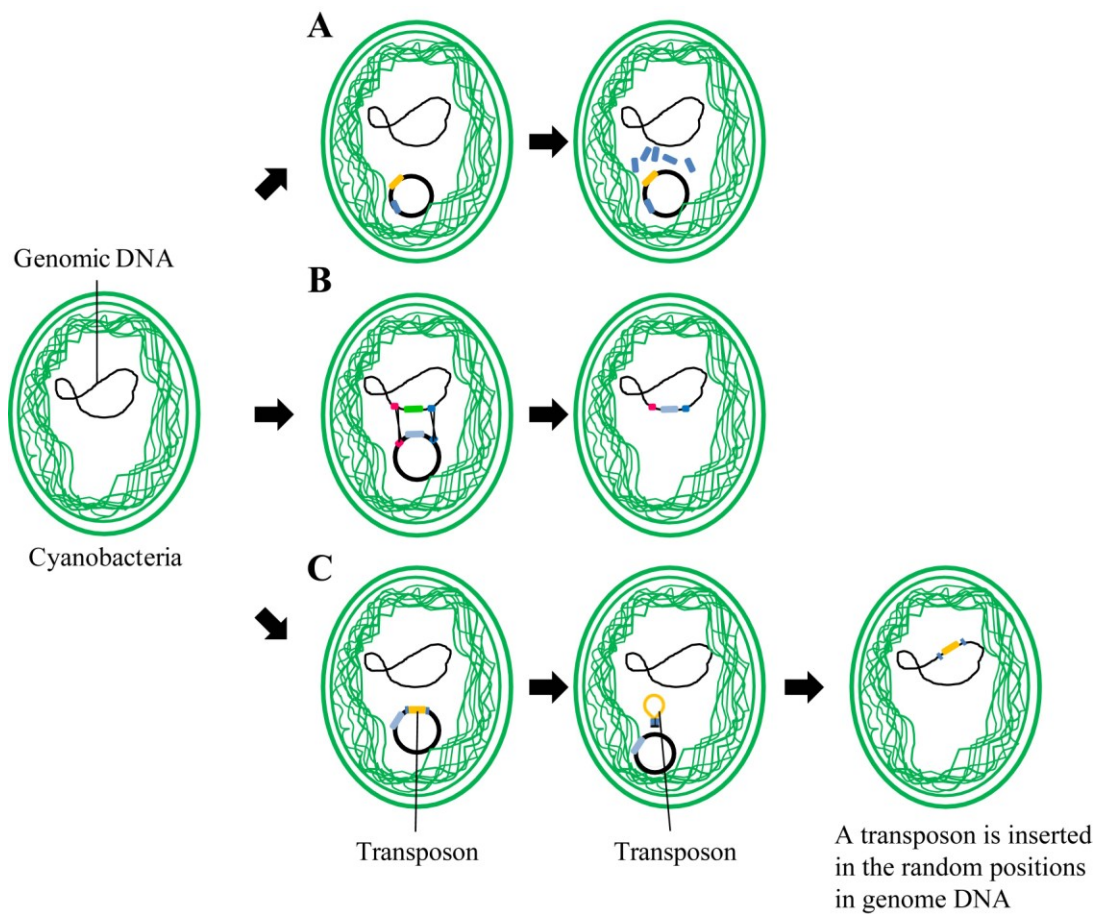


Fig. 7 Genetic recombination technology of cyanobacteria

(A) Gene transfer system. (B) Gene targeting system. (C) Comprehensive gene disruption. Gene transfer system (A) is expression of target gene by gene transfer. Gene targeting system (B) is gene disruption or/and genetic modification by homologous recombination. Comprehensive gene disruption (C) is method to screen transformants from a phenotype and to identify a causative gene.

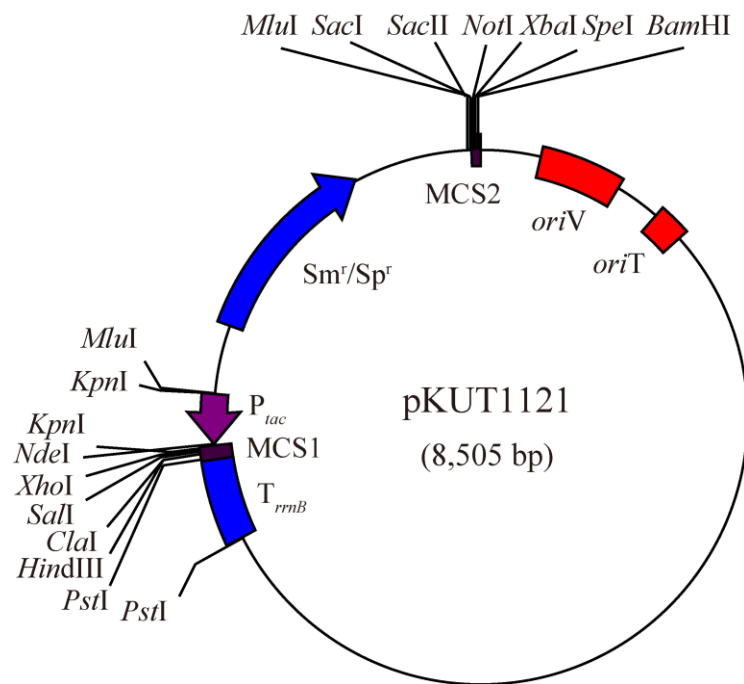


Fig. 8 The vector map of pKUT1121

pKUT1121 is derived from a broad-host-range plasmid RSF1010. The streptomycin resistant gene cassette was introduced into pKUT1121 as an antibacterial resistance gene. P_{lac} , *tac* promoter of *E. coli*; T_{rrnB} , transfer terminator of *rrnB* which is one of the *E. coli* ribosomal RNAs; His₆, His-tag; *oriV*, replication origin of plasmid; *oriT*, origin of conjugational transfer. When this vector is digested by *MluI* or *XbaI*, the fragments are 1,950 bp and 6,555 bp, 8,505 bp, respectively.

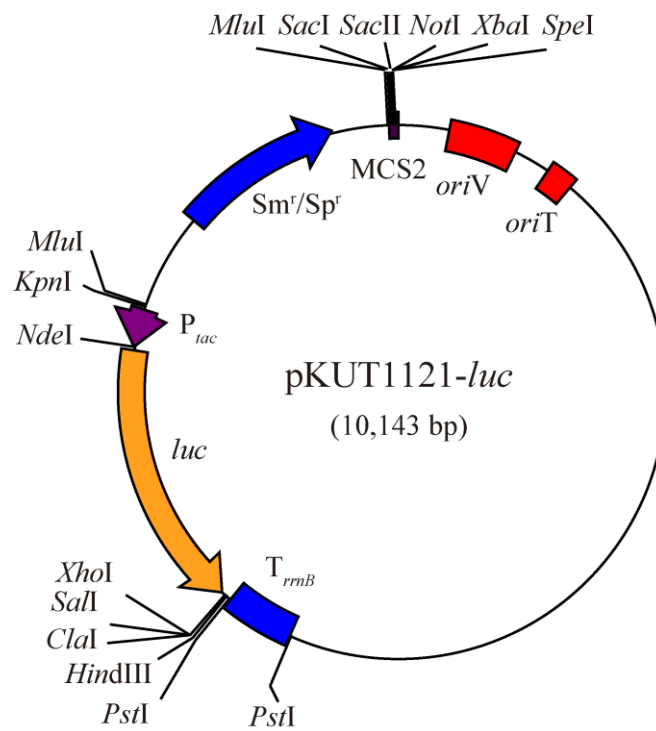


Fig. 9 The vector map of pKUT1121-*luc*

pKUT1121-*luc* was constructed that firefly luciferase (*luc*) gene was introduced into *Nde*I and *Xho*I site of pKUT1121. When this vector is digested by *Nde*I and *Xho*I, the fragments are 1,659 bp and 8,484 bp.

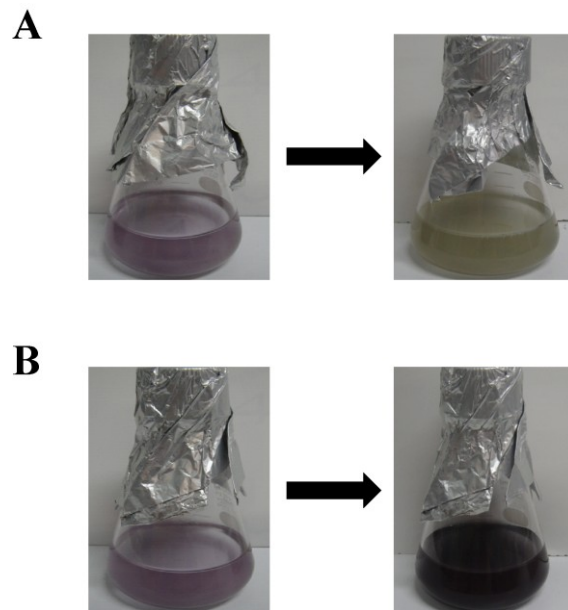


Fig. 10 Cultivation of *G. violaceus* wild type

Left, immediately after inoculation of *G. violaceus* wild type; right, the 25 days after cultivation. (A) Cultivation on the conditions of $15 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, 28°C . (B) Cultivation on the conditions of $5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, 25°C . The cultivation was without shaking, only once shook when these photographs were taken. The color degradation of cells was observed in (A) and multiplication of the cells was observed in (B).

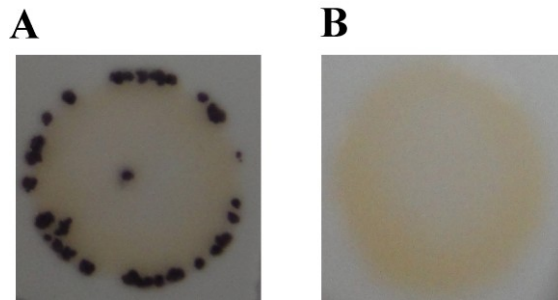


Fig. 11 Sm resistant colonies formed by conjugation

(A) Conjugation of *G. violaceus* and XL1-Blue MRF' (pRK2013, pKUT1121). (B) Wild type *G. violaceus* as a negative control.

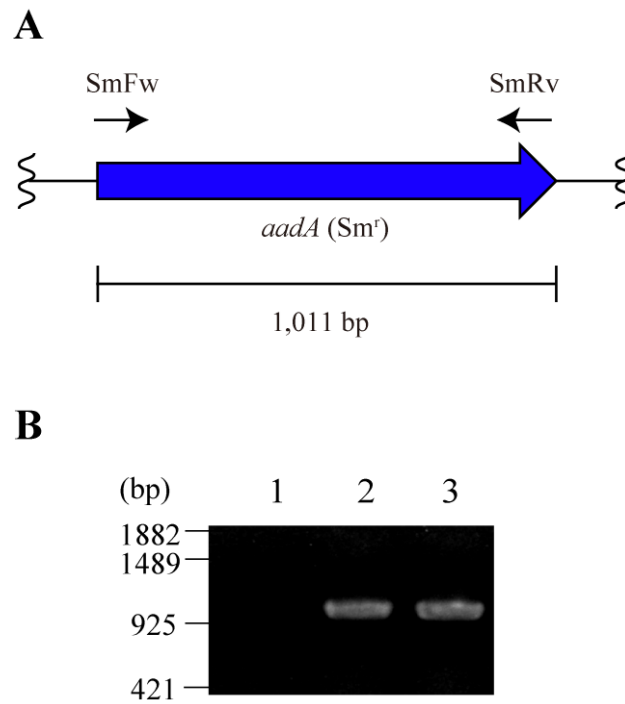


Fig. 12 Confirmation of the production of *G. violaceus* transformants

(A) The amplified region by PCR in pKUT1121. (B) Coding regions of *aadA* was amplified by PCR. Templates for PCR are as follows: lane 1, total DNA prepared from wild type *G. violaceus*; lane 2, pKUT1121 prepared from *E.coli*; lane 3, total DNA prepared from pKUT1121 strain.

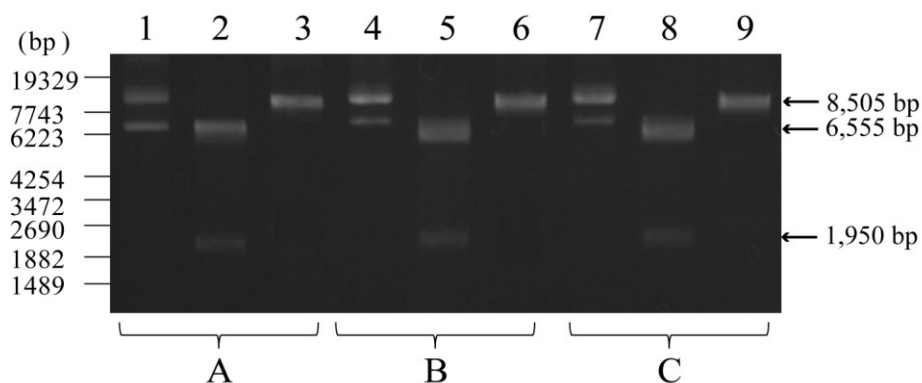


Fig. 13 Restriction enzyme digestion for examining whether the introduced plasmid exists in the exconjugant as a plasmid

(A) Original plasmid. (B) The plasmid prepared from *E. coli* DH5 α transformed by total DNA prepared from pKUT1121 strain. (C) The plasmid prepared from *E. coli* XL1-Blue MRF' transformed by total DNA prepared from pKUT1121 strain. Lanes 1, 4 and 7; not digested plasmid, Lanes 2, 5 and 8; digested with *Mlu*I, Lanes 3, 6 and 9; digested with *Xba*I. The chain lengths of pKUT1121 are 1,950 bp and 6,555 bp, when it digests with *Mlu*I. The chain length of pKUT1121 is 8,505 bp, when it digests with *Xba*I.

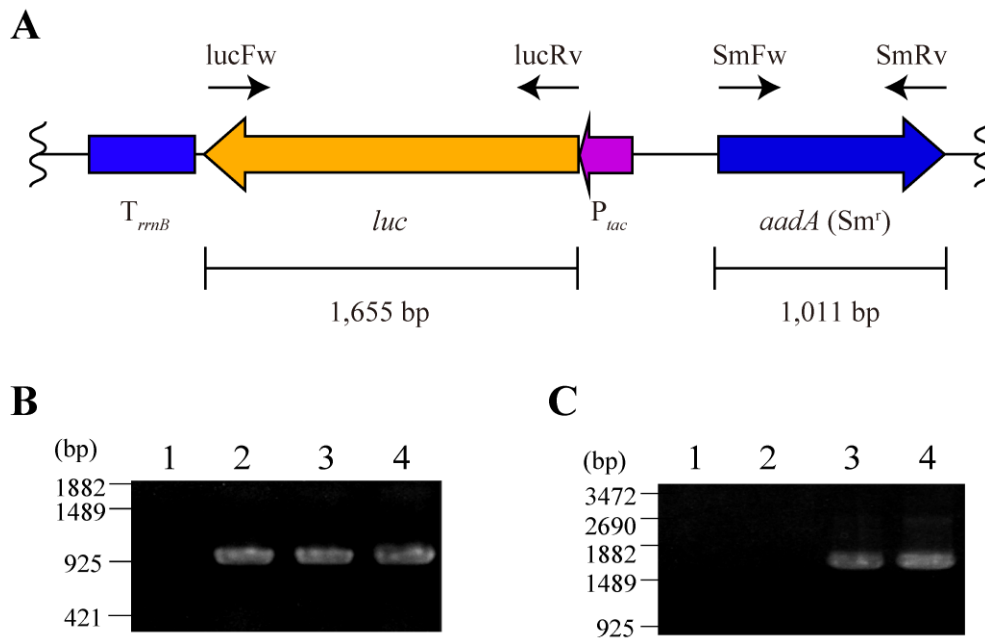


Fig. 14 Confirmation of the production of *G. violaceus* transformants

(A) The amplified region by PCR in pKUT1121-*luc*. Coding regions of *aadA* (B) and *luc* (C) were amplified by PCR. Templates for PCR are as follows: lane 1, total DNA prepared from wild type *G. violaceus*; lane 2, total DNA prepared from pKUT1121 strain; lane 3, pKUT-*luc* prepared from *E. coli*; lane 4, total DNA prepared from pKUT-*luc* strain.

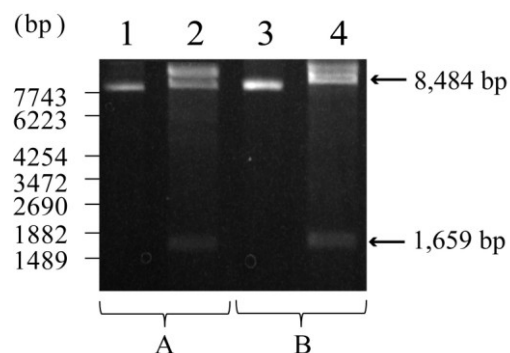


Fig. 15 Restriction enzyme digestion for examining whether the introduced plasmid exists in the exconjugant as a plasmid

(A) Original plasmid. (B) The plasmid prepared from *E. coli* XL1-Blue MRF' transformed by total DNA prepared from pKUT1121-*luc* strain. Lanes 1 and 3; not digested plasmid, Lanes 2 and 4; digested with *Nde*I and *Xho*I. The chain lengths of pKUT1121-*luc* are 1,659 bp and 8,484 bp, when it digests with *Nde*I and *Xho*I.

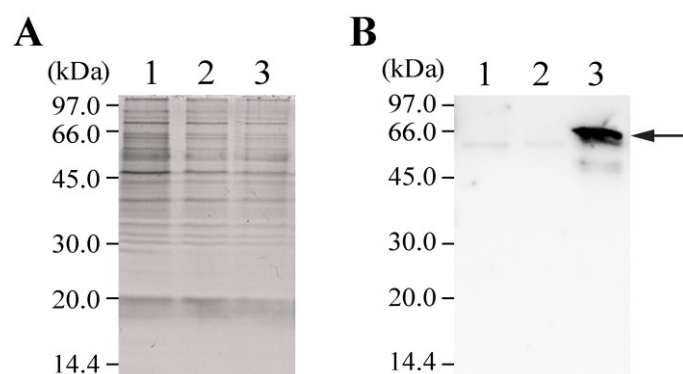


Fig. 16 Detection of luciferase by Western blotting

Total proteins were used for SDS-PAGE and Western blotting. Lane 1, wild type *G. violaceus*; lane 2, pKUT1121 strain; lane 3, pKUT1121-*luc* strain. (A) The gel image of CBB staining after SDS-PAGE. (B) The result of Western blotting using anti-luciferase antibody. The arrow indicates the position of luciferase in the membrane.

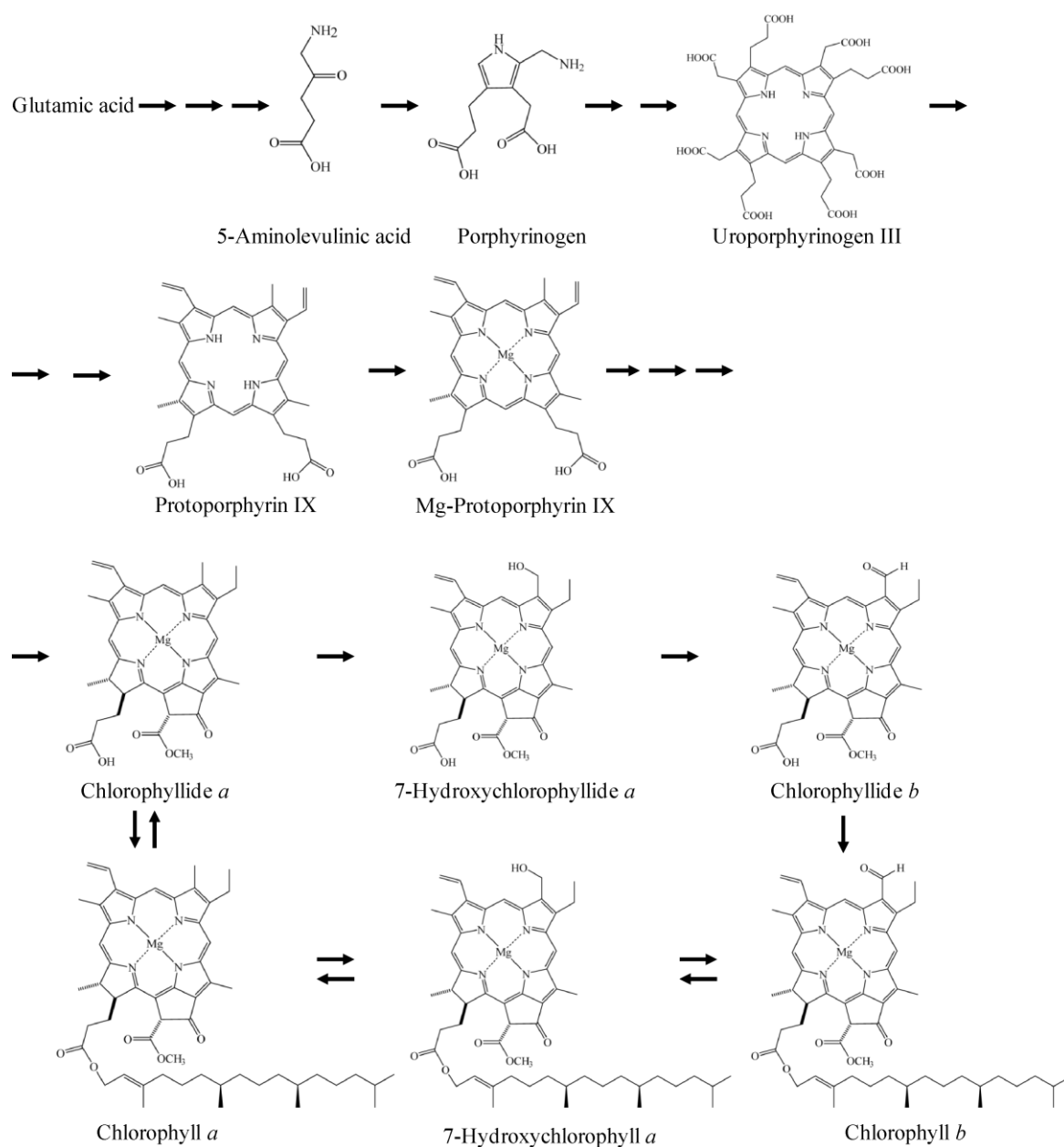


Fig. 17 Chlorophyll biosynthetic pathway

Chlorophyll is biosynthesized through the reaction of a multistep process by using glutamic acid as a start substance. Chl *a* is synthesized from chlorophyllide *a*. Chl *b* is synthesized from chlorophyllide *a* through chlorophyllide *b*. CAO catalyzes the conversion from chlorophyllide *a* to chlorophyllide *b*.

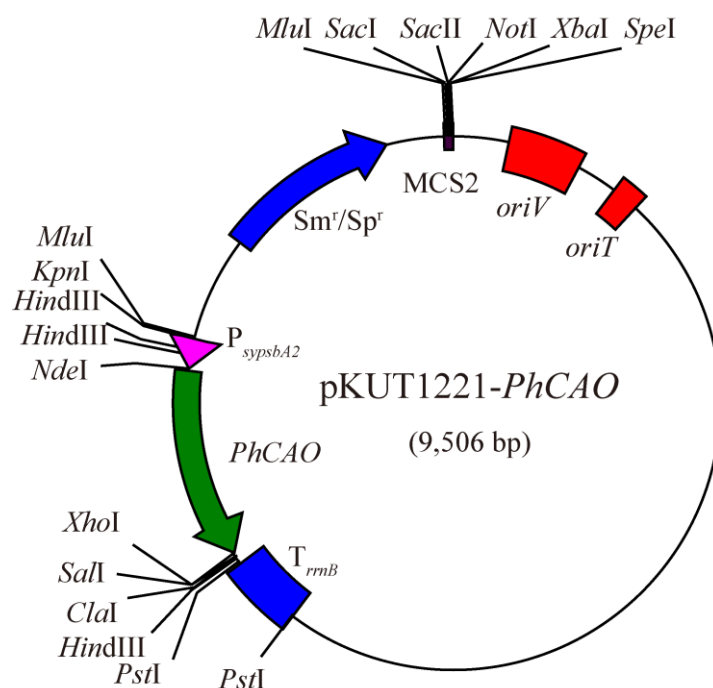


Fig. 18 The vector map of pKUT1221-*PhCAO*

pKUT1221-*PhCAO* was constructed that the promoter region of the expression vector with *PhCAO* gene, pKUT1321-*PhCAO* (Tsuchiya et al. 2012B), was replaced by the promoter region of *Synechocystis psbA2* gene. *PhCAO* is *CAO* gene from *Prochlorothrix hollandica*.

When this vector is digested by *XhoI*, *MluI*, or *NdeI* and *XhoI* the fragments are 9,506 bp, 7,556 bp and 1,950 bp, 8,402 bp and 1,104 bp, respectively.

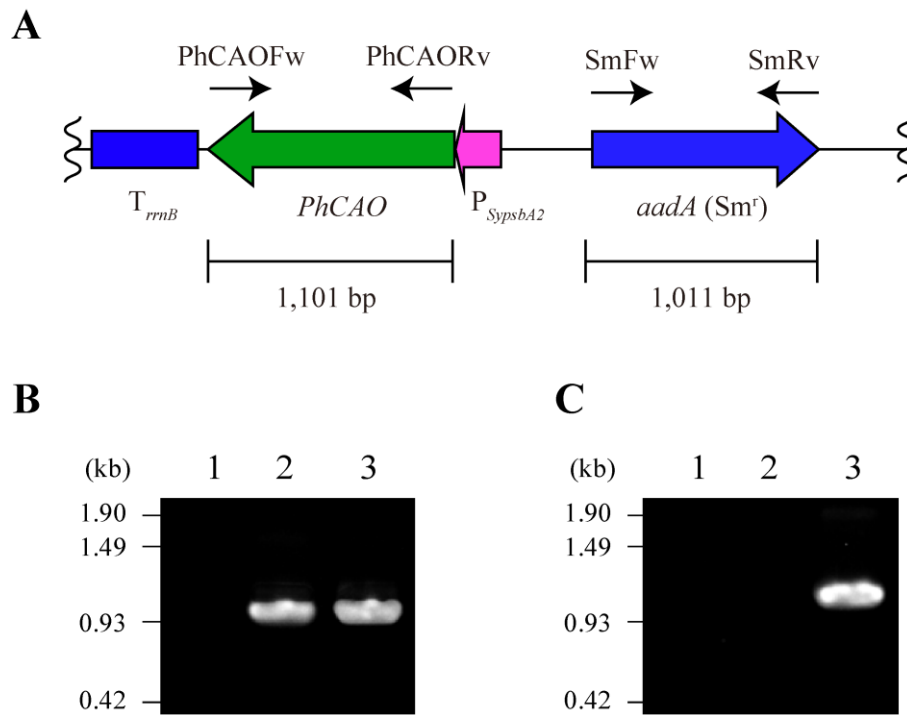


Fig. 19 Confirmation of the production of *G. violaceus* transformant

(A) The amplified region by PCR in pKUT1221-*PhCAO*. Coding regions of *aadA* (B) and *PhCAO* (C) were amplified by PCR. Templates for PCR are as follows: lane 1, total DNA from wild type *G. violaceus*; lane 2, total DNA from the pKUT1121 strain; lane 3, total DNA from the Sm-resistant exconjugant.

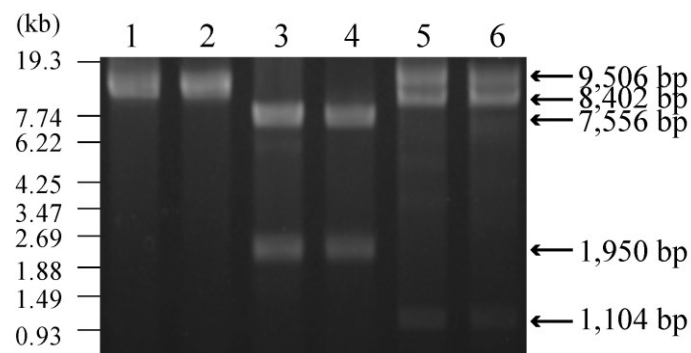


Fig. 20 Restriction enzyme digestion for examining whether the introduced plasmid exists in the exconjugant as a plasmid

Lanes 1, 3 and 5, original plasmid; lanes 2, 4 and 6; the plasmid prepared from *E. coli* transformed with total DNA prepared from the pKUT1221-*PhCAO* strain. Lanes 1 and 2, plasmid digested with *Xho*I; Lanes 3 and 4, plasmid digested with *Mlu*I; Lanes 5 and 6, plasmid digested with *Nde*I and *Xho*I.

The chain length of pKUT1221-*PhCAO* is 9,506 bp, when it digests with *Xho*I. The chain lengths of pKUT1221-*PhCAO* are 7,556 bp and 1,950 bp, when it digests with *Mlu*I. The chain lengths of pKUT1221-*PhCAO* are 8,402 bp and 1,104 bp, when it digests with *Nde*I and *Xho*I.

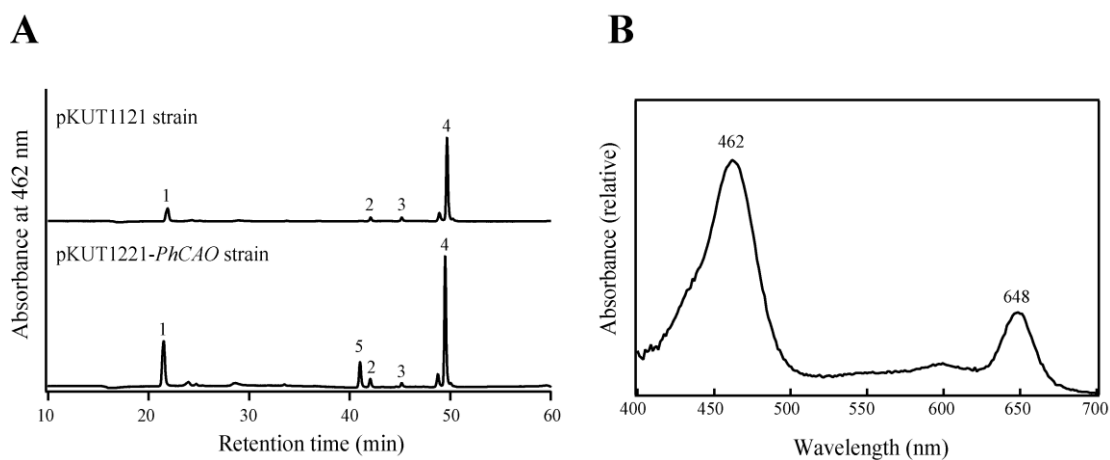


Fig. 21 Pigment composition of *G. violaceus* transformant cells analyzed by HPLC

(A) HPLC chromatogram of the methanol extract from the pKUT1121 strain and the pKUT1221-*PhCAO* strain monitored at 462 nm. HPLC chromatograms are normalized by the content of total Chl. 1, (2*S*,2'*S*)-oscillol 2,2'-di(α -L-fucoside); 2, echinenone; 3, Chl *a*; 4, β -carotene; 5, Chl *b*. (B) Absorption spectrum of peak 5 measured by photodiode array detector. Absorption maximum wavelengths of Soret and Qy bands are indicated.

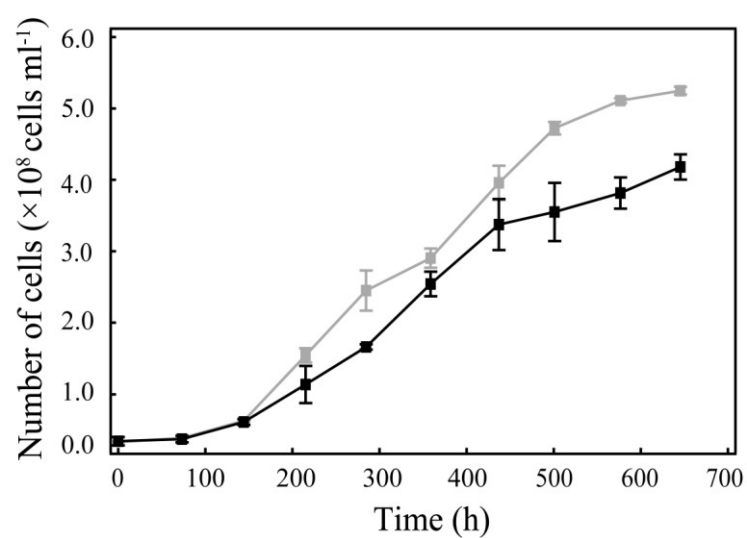


Fig. 22 Growth curves of *G. violaceus* transformants monitored by cell number

Gray squares, pKUT1121 strain; black squares, pKUT1221-*PhCAO* strain. Error bars represent the standard deviations of triplicate measurements. These strains were cultivated at 25°C under the continuous light (10 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$).

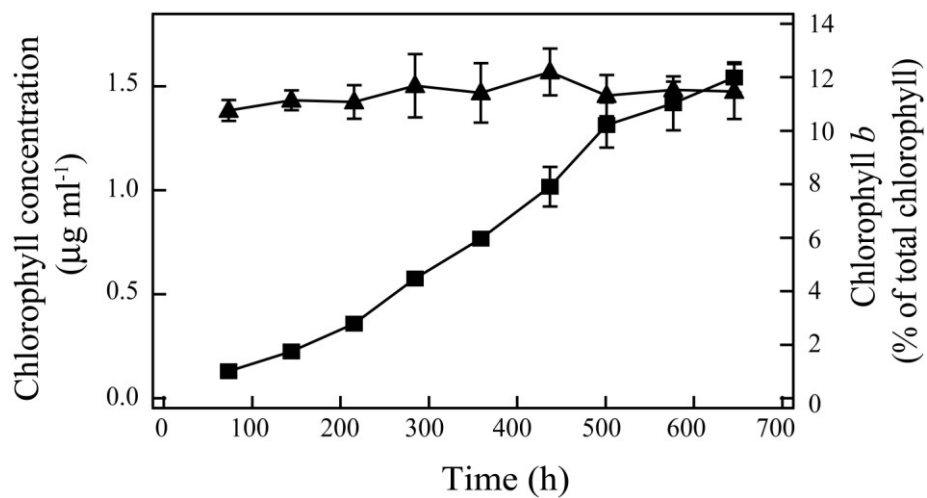


Fig. 23 Molar ratio of Chl *b* to total Chl in the pKUT1221-*PhCAO* strain during cell growth

Cells grown under the continuous light were harvested at the indicated times. Closed squares, Chl concentration per milliliter culture; closed triangles, Chl *b* mol percent ratio. Error bars represent the standard deviations of triplicate measurements.

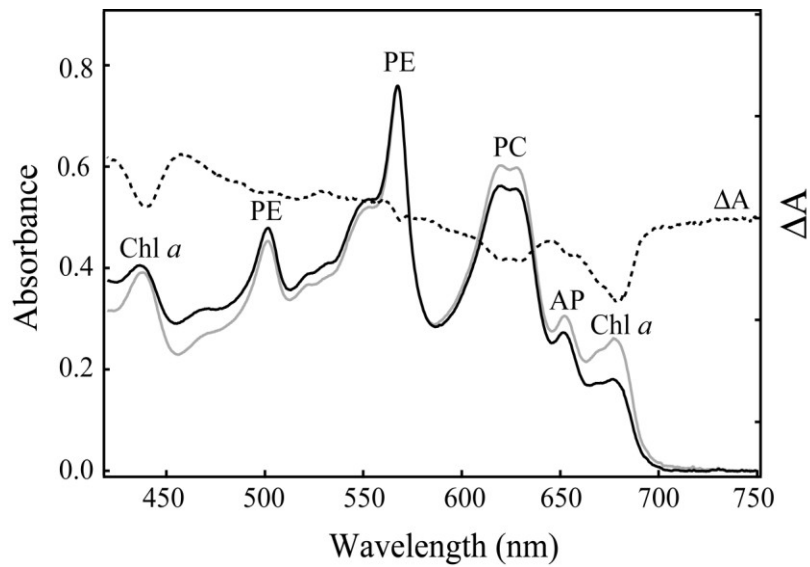


Fig. 24 Absorption spectra of *G. violaceus* transformant cells at 80 K

Gray line, pKUT1121 strain; black line, pKUT1221-*PhCAO* strain; black broken line, difference absorption spectrum (ΔA). AP, allophycocyanin; PC, phycocyanin; PE, phycoerythrin. Absorption spectra were normalized at the absorption maximum wavelength of PE.

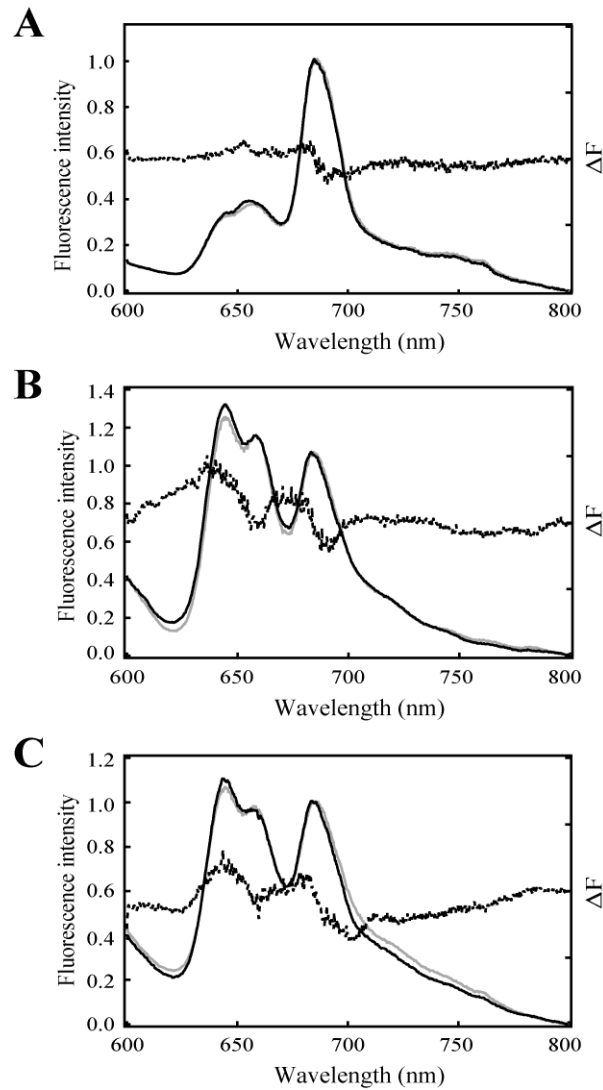


Fig. 25 Fluorescence spectra of *G. violaceus* transformant cells at 77 K

(A) The fluorescence spectra excited at 440 nm which is an excitation wavelength of Chl *a*. (B) The fluorescence spectra excited at 560 nm which is an excitation wavelength of phycoerythrin. (C) The fluorescence spectra excited at 458 nm which is a peak wavelength of low-temperature difference absorption spectrum. Gray line, pKUT1121 strain; black line, pKUT1221-*PhCAO* strain; black broken line, difference fluorescence spectrum (ΔF). The fluorescence spectra are normalized at the peak emission wavelength of Chl *a*.

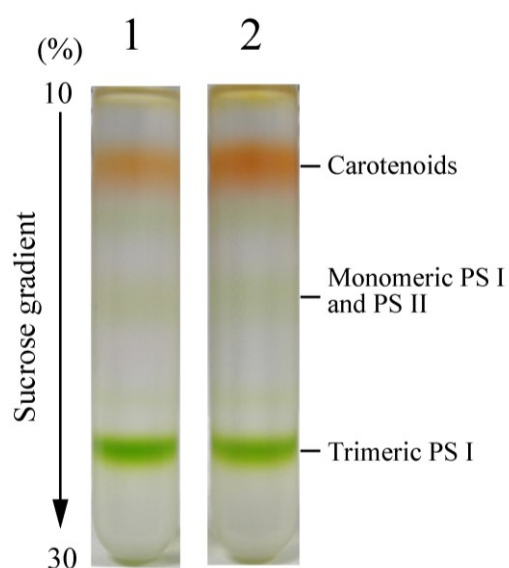


Fig. 26 Sedimentation pattern of solubilized proteins in the sucrose density-gradient (10–30%) centrifugation

(1) Preparation from the pKUT1121 strain. (2) Preparation from the pKUT1221-*PhCAO* strain. Samples loaded on the sucrose density-gradient were prepared by the solubilization of cell membrane from each strain with *n*-dodecyl β -D-maltoside. Samples of equal Chl amount (32.5 μ g of total Chl) were used for separation.

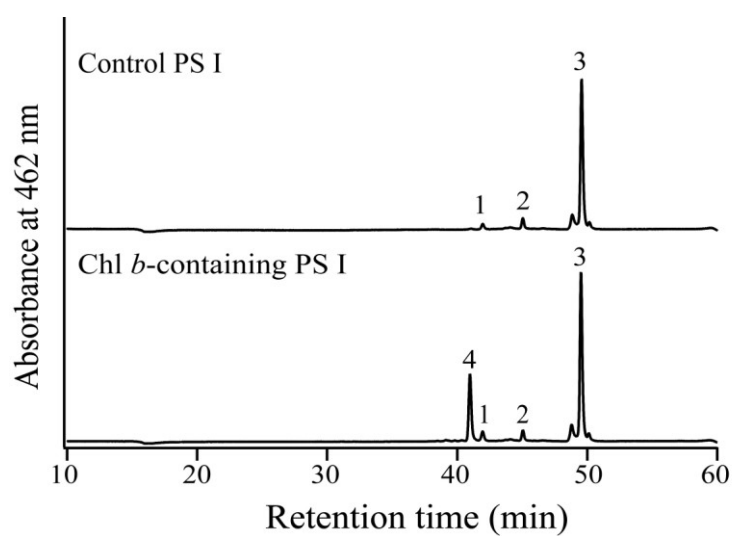


Fig. 27 Pigment composition of the PS I complexes isolated from *G. violaceus* transformants analyzed by HPLC

HPLC chromatogram of the methanol extract from the control PS I and the Chl *b*-containing PS I monitored at 462 nm. HPLC chromatograms are normalized by the content of total Chl. 1, echinenone; 2, Chl *a*; 3, β -carotene; 4, Chl *b*.

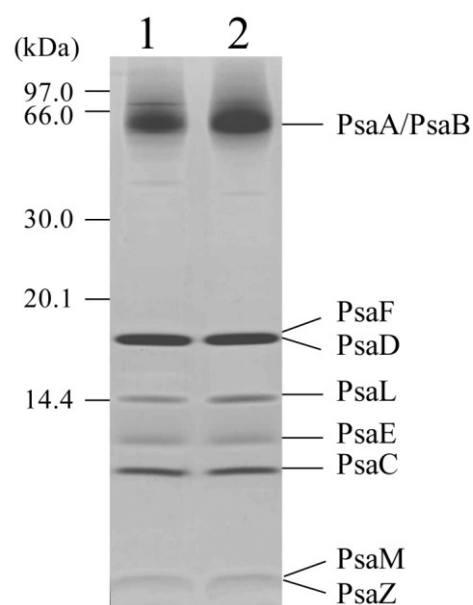


Fig. 28 SDS-PAGE of PS I complexes isolated from *G. violaceus* transformants

Lane 1, control PS I; lane 2, Chl *b*-containing PS I. Sample of 4 μg total Chl were used for separation.

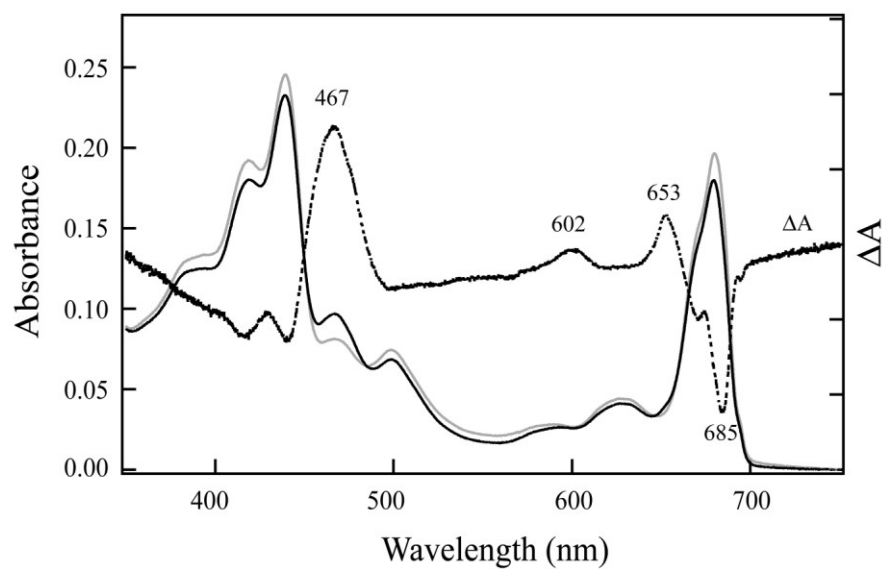


Fig. 29 Absorption spectra of the PS I complexes isolated from *G. violaceus* transformants at 80 K

Gray line, control PS I; black line, Chl *b*-containing PS I; black broken line, difference absorption spectrum (ΔA). These samples were measured at the same total Chl concentration.

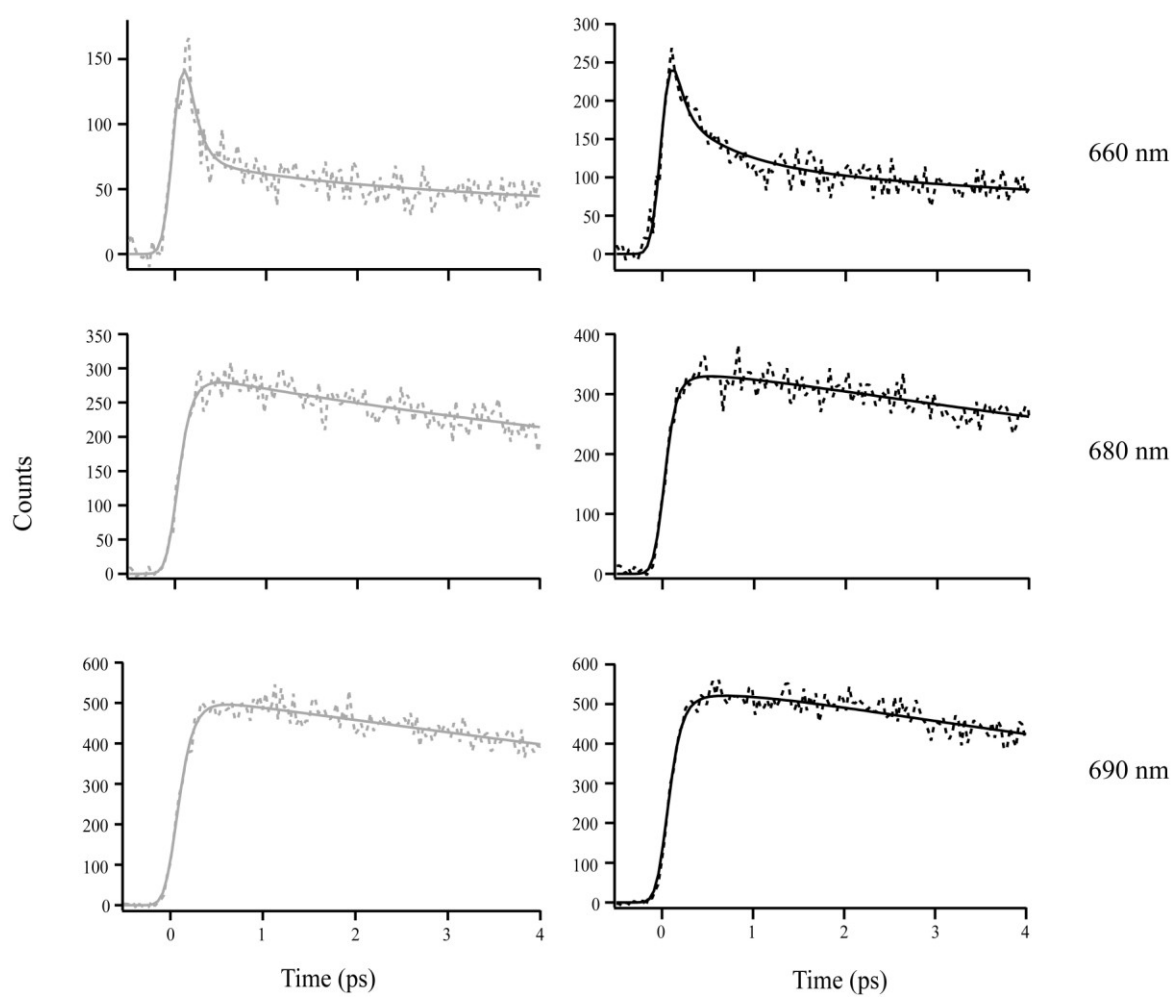


Fig. 30 The fluorescence rise and decay curves in femtosecond to picosecond region at room temperature

Gray line, control PS I; black line, Chl *b*-containing PS I.

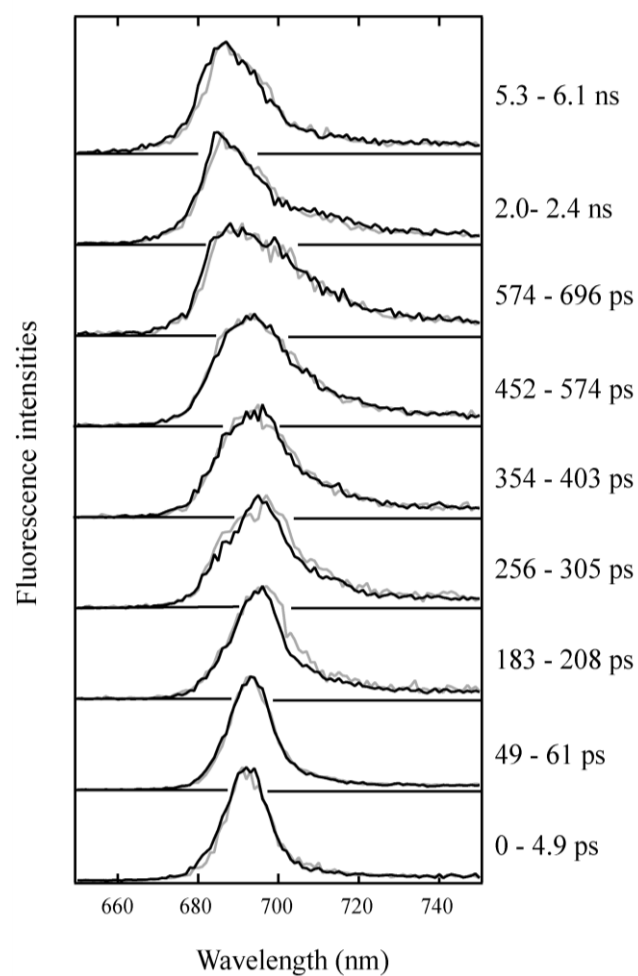


Fig. 31 The normalized time-resolved fluorescence spectra of the PS I complexes isolated from *G. violaceus* transformants at 77 K

Gray line, control PS I; black line, Chl *b*-containing PS I.

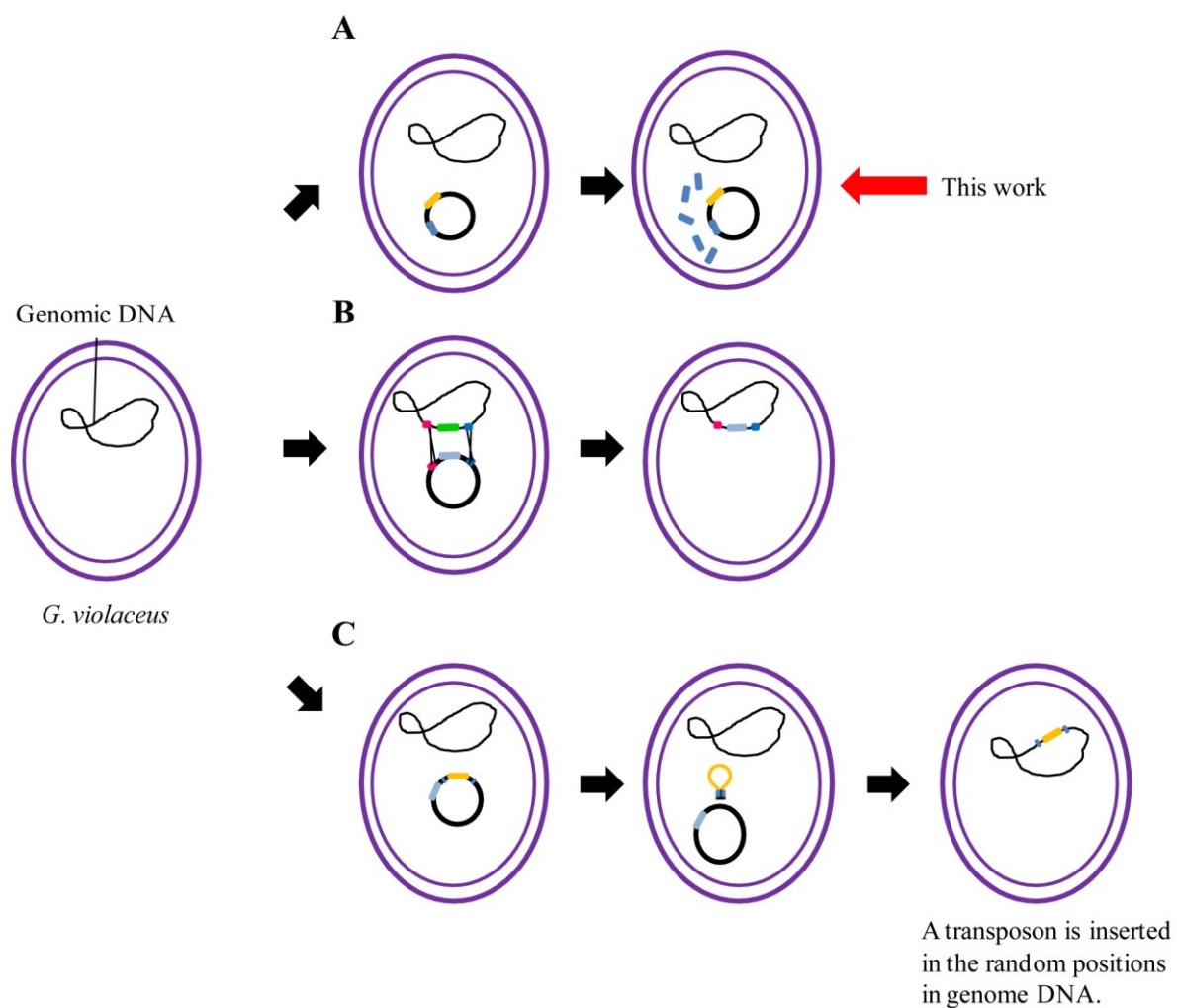


Fig. 32 The experimental system which should be developed for the molecular genetic analysis of *G. violaceus* and the experimental system developed in this study

In this study, I developed gene transfer system (A, red arrow), and I succeeded in expressing *luc* gene and *PhCAO* gene by developed transformation system. For further research of *G. violaceus*, development of gene targeting system (B) and comprehensive destruction of a gene system (C) are also required.